

USE OF RECOMBINANT PARAINFLUENZA VIRUSES (PIVs) AS VECTORS TO PROTECT AGAINST INFECTION AND DISEASE CAUSED BY PIV AND OTHER HUMAN PATHOGENS

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CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/170,195, filed by Murphy et al. on December 10, 1999, the disclosure of which is incorporated by reference.

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BACKGROUND OF THE INVENTION

Human parainfluenza virus type 3 (HPIV3) is a common cause of serious lower respiratory tract infection in infants and children less than one year of age. It is second only to respiratory syncytial virus (RSV) as a leading cause of hospitalization for viral lower respiratory tract disease in this age group (Collins et al., 3rd ed. In "Fields Virology," B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996; Crowe et al., Vaccine 13:415-421, 1995; Marx et al., J. Infect. Dis. 176:1423-1427, 1997). Infections by this virus results in substantial morbidity in children less than 3 years of age. HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup) and also can cause severe pneumonia and bronchiolitis (Collins et al., 3rd ed. In "Fields Virology," B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996). In a long term study over a 20-year period, HPIV1, HPIV2, and HPIV3 were identified as etiologic agents for 6.0, 3.2, and 11.5%, respectively, of hospitalizations for respiratory tract disease accounting in total for 18% of the hospitalizations, and, for this reason, there is a need for an effective vaccine (Murphy et al., Virus Res. 11:1-15, 1988). The parainfluenza viruses have also been identified in a significant proportion of cases of virally-induced middle ear effusions in children with otitis media (Heikkinen et al., N. Engl. J. Med. 340:260-4, 1999). Thus, there is a need to produce a vaccine against these viruses that can prevent the serious lower respiratory tract disease and the otitis media that accompanies these HPIV infections. HPIV1, HPIV2, and HPIV3 are distinct serotypes which do not elicit significant cross-protective immunity.

To facilitate development of PIV vaccine candidates, recombinant DNA technology has recently made it possible to recover infectious negative-stranded RNA viruses from cDNA (for reviews, see Conzelmann, *J. Gen. Virol.* 77:381-89, 1996; Palese et al., *Proc. Natl. Acad. Sci. U.S.A.* 93:11354-58, 1996). In this context, recombinant rescue has been reported for infectious respiratory syncytial virus (RSV), rabies virus (RaV), simian virus 5 (SV5), rinderpest virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles virus (MeV), and Sendai virus (SeV) from cDNA-encoded antigenomic RNA in the presence of essential viral proteins (see, e.g., Garcin et al., *EMBO J.* 14:6087-6094, 1995; Lawson et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:4477-81, 1995; Radecke et al., *EMBO J.* 14:5773-5784, 1995; Schnell et al., *EMBO J.* 13:4195-203, 1994; Whelan et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:8388-92, 1995; Hoffman et al., *J. Virol.* 71:4272-4277, 1997; Kato et al., *Genes to Cells* 1:569-579, 1996; Roberts et al., *Virology* 247:1-6, 1998; Baron et al., *J. Virol.* 71:1265-1271, 1997; International Publication No. WO 97/06270; Collins et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:11563-11567, 1995; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to published International Application No. WO 98/02530 and priority U.S. Provisional Application Nos. 60/047,634, filed May 23, 1997, 60/046,141, filed May 9, 1997, and 60/021,773, filed July 15, 1996); U.S. Patent Application No. 09/291,894, filed on April 13, 1999; International Application No. PCT/US00/09695, filed April 12, 2000 (which claims priority to U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999); International Application No. PCT/US00/17755, filed June 23, 2000 (which claims priority to U.S. Provisional Patent Application Serial No. 60/143,132, filed by Bucholz et al. on July 9, 1999); Juhasz et al., *J. Virol.* 71:5814-5819, 1997; He et al. *Virology* 237:249-260, 1997; Peters et al. *J. Virol.* 73:5001-5009, 1999; Baron et al. *J. Virol.* 71:1265-1271, 1997; Whitehead et al., *Virology* 247:232-9, 1998a; Whitehead et al., *J. Virol.* 72:4467-4471, 1998b; Jin et al. *Virology* 251:206-214, 1998; Bucholz et al. *J. Virol.* 73:251-259, 1999; and Whitehead et al., *J. Virol.* 73:3438-3442, 1999, each incorporated herein by reference in its entirety for all purposes).

In more specific regard to the instant invention, a method for producing HPIV with a wt phenotype from cDNA was recently developed for recovery of infectious, recombinant HPIV3 JS strain (see, e.g., Durbin et al., *Virology* 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998 (corresponding to U.S. Provisional Application No. 60/059,385, filed September 19, 1997); and U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), each incorporated herein by reference). In addition, these disclosures

allow for genetic manipulation of viral cDNA clones to determine the genetic basis of phenotypic changes in biological mutants, e.g., which mutations in the HPIV3 *cp45* virus specify its *ts*, *ca* and *att* phenotypes, and which gene(s) or genome segment(s) of BPIV3 specify its attenuation phenotype. Additionally, these and related disclosures render it feasible to construct novel PIV vaccine candidates having a wide range of different mutations and to evaluate their level of attenuation, immunogenicity and phenotypic stability (see also, U.S. Application No. 09/586,479, filed June 1, 2000 (corresponding to U.S. Provisional Patent Application Serial No. 60/143,134, filed on July 9, 1999); and U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, each incorporated herein by reference).

Thus, infectious wild type recombinant PIV3, (r)PIV3, as well as a number of *ts* derivatives, have now been recovered from cDNA, and reverse genetics systems have been used to generate infectious virus bearing defined attenuating mutations and to study the genetic basis of attenuation of existing vaccine viruses. For example, the three amino acid substitutions found in the L gene of *cp45*, singularly or in combination, have been found to specify the *ts* and attenuation phenotypes. Additional *ts* and attenuating mutations are present in other regions of the PIV3*cp45*. In addition a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45*L (Skiadopoulos et al., J. Virol. 72:1762-8, 1998; Tao et al., J. Virol. 72:2955-2961, 1998; Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.*cp45*L was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. Yet another recombinant chimeric virus, designated rPIV3-1.*cp45*, has been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations that occur in HN and F. This recombinant vaccine candidate is highly attenuated in the upper and lower respiratory tract of hamsters and induces a high level of protection against HPIV1 infection (Skiadopoulos et al., Vaccine 18:503-510, 1999).

Recently, a number of studies have focused on the possible use of viral vectors to express foreign antigens toward the goal of developing vaccines against a pathogen for which other vaccine alternatives are not proved successful. In this context, a number of reports suggest that foreign genes may be successfully inserted into a recombinant negative strand RNA virus genome or antigenome with varying effects (Bukreyev et al., J.

necessary to develop a viable vaccination strategy, including an immunogenic and efficacious route of administration.

The three human mononegaviruses that are currently being considered as vaccine vectors, namely measles, mumps, and rabies viruses, have additional limitations that combine to make them weak candidates for further development as vectors. For example, measles virus has been considered for use as a vector for the protective antigen of hepatitis B virus (Singh et al., J. Virol. 73:4823-8, 1999). However, this combined measles virus-hepatitis B virus vaccine could only be given, like the licensed measles virus vaccine, after nine months of age, whereas the current hepatitis B virus vaccine is recommended for use in early infancy. This is because the currently licensed measles virus vaccine is administered parenterally and is very sensitive to neutralization and immunosuppression by maternal antibodies, and therefore is not effective if administered before 9-15 months of age. Thus, it could not be used to vector antigens that cause disease in early infancy and therefore would not be useful for viruses such as RSV and the HPIVs. Another well known, characteristic effect of measles virus infection is virus-mediated immunosuppression, which can last several months. Immunosuppression would not be a desirable feature for a vector. The attenuated measles virus vaccine was associated with altered immune responses and excess mortality when administered at increased dose, which might be due at least in part to virus-induced immunosuppression and indicates that even an attenuated measles virus might not be appropriate as a vector. Furthermore, the use of measles virus as a vector would be inconsistent with the global effort to eradicate this pathogen. Indeed, for these reasons it would be desirable to end the use of live measles virus and replace the present measles virus vaccine with a PIV vector that expresses measles virus protective antigens, as described herein.

Rabies virus, a rare cause of infection of humans, has been considered for use as a vector (Mebatsion et al., Proc. Natl. Acad. Sci. USA 93:7310-4, 1996), but it is unlikely that a vector that is 100% fatal for humans would be developed for use as a live attenuated virus vector, especially since immunity to the rabies virus, which is not a ubiquitous human pathogen, is not needed for the general population. While mumps and measles viruses are less pathogenic, infection by either virus can involve undesirable features. Mumps virus infects the parotid gland and can spread to the testes, sometimes resulting in sterility. Measles virus establishes a viremia, and the widespread nature of its infection is exemplified by the associated widespread rash. Mild encephalitis during mumps and measles infection is

not uncommon. Measles virus also is associated with a rare progressive fatal neurological disease called subacute sclerosing encephalitis. In contrast, PIV infection and disease in normal individuals is limited to the respiratory tract, a site that is much more advantageous for immunization than the parental route. Viremia and spread to second sites can occur in severely immunocompromised experimental animals and humans, but this is not a characteristic of the typical PIV infection. Acute respiratory tract disease is the only disease associated with PIVs. Thus, use of PIVs as vectors will, on the basis of their biological characteristics, avoid complications such as interaction of virus with peripheral lymphocytes, leading to immunosuppression, or infection of secondary organs such as the testes or central nervous system, leading to other complications.

Among a host of human pathogens for which a vector-based vaccine approach may be desirable is the measles virus. A live attenuated vaccine has been available for more than three decades and has been largely successful in eradicating measles disease in the United States. However, the World Health Organization estimates that more than 45 million cases of measles still occur annually, particularly in developing countries, and the virus contributes to approximately one million deaths per year.

Measles virus is a member of the *Morbillivirus* genus of the *Paramyxoviridae* family (Griffin et al., In “Fields Virology”, B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). It is one of the most contagious infectious agents known to man and is transmitted from person to person via the respiratory route (Griffin et al., In “Fields Virology”, B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). The measles virus has a complex pathogenesis, involving replication in both the respiratory tract and various systemic sites (Griffin et al., In “Fields Virology”, B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996).

Although both mucosal IgA and serum IgG measles virus-specific antibodies can participate in the control of measles virus, the absence of measles virus disease in very young infants possessing maternally-acquired measles virus-specific antibodies identifies serum antibodies as a major mediator of resistance to disease (Griffin et al., In “Fields

Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). The two measles virus glycoproteins, the hemagglutinin (HA) and fusion (F) proteins, are the major neutralization and protective antigens (Griffin et al., In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996).

The currently available live attenuated measles vaccine is administered by a parenteral route (Griffin et al., In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). Both the wild type measles virus and the vaccine virus are very readily neutralized by antibodies, and the measles virus vaccine is rendered non-infectious by even very low levels of maternally-acquired measles virus-specific neutralizing antibodies (Halsey et al., N. Engl. J. Med. 313:544-9, 1985; Osterhaus et al., Vaccine 16:1479-81, 1998). Thus, the vaccine virus is not given until the passively-acquired maternal antibodies have decreased to undetectable levels. In the United States, measles virus vaccine is not given until 12 to 15 months of age, a time when almost all children are readily infected with the measles virus vaccine. In the developing world, measles virus continues to have a high mortality rate, especially in children within the latter half of the first year of life (Gellin et al., J. Infect. Dis. 170:S3-14, 1994; Taylor et al., Am. J. Epidemiol. 127:788-94, 1988). This occurs because the measles virus, which is highly prevalent in these regions, is able to infect that subset of infants in whom maternally-acquired measles virus-specific antibody levels have decreased to a non-protective level. Therefore, there is a need for a measles virus vaccine that is able to induce a protective immune response even in the presence of measles virus neutralizing antibodies with the goal of eliminating measles virus disease occurring within the first year of life as well as that which occurs thereafter. Given this need, there have been numerous attempts to develop an immunization strategy to protect infants in the latter half of the first year of life against measles virus, but none of these strategies has been effective to date.

The first strategy for developing an early measles vaccine involved administration of the licensed live attenuated measles virus vaccine to infants about six months of age by one of the following two methods (Cutts et al., Biologicals 25:323-38, 1997). In one general protocol, the live attenuated measles virus was administered

young uncooperative infant (Cutts et al., Biologicals 25:323-38, 1997). In another attempt to immunize six-month old infants, the measles vaccine virus was administered parenterally at a 10- to 100-fold increased dose (Markowitz et al., N. Engl. J. Med. 322:580-7, 1990). Although high-titer live measles vaccination improved seroconversion in infants 4-6 months of age, there was an associated increase in mortality in the high-titer vaccine recipients later in infancy (Gellin et al., J. Infect. Dis. 170:S3-14, 1994; Holt et al., J. Infect. Dis. 168:1087-96, 1993; Markowitz et al., N. Engl. J. Med. 322:580-7, 1990) and this approach to immunization has been abandoned.

A second strategy previously explored for a measles virus vaccine was the use of an inactivated measles virus vaccine, specifically, a formalin inactivated whole measles virus or a subunit virus vaccine prepared from measles virus (Griffin et al., In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). However, the clinical use of the vaccines in the 1960's revealed a very serious complication, namely, that the inactivated virus vaccines potentiated disease rather than prevented it (Fulginiti et al., JAMA 202:1075-80, 1967). This was first observed with formalin-inactivated measles virus vaccine (Fulginiti et al., JAMA 202:1075-80, 1967). Initially, this vaccine prevented measles, but after several years vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia (Fulginiti et al., JAMA 202:1075-80, 1967; Nader et al., J. Pediatr. 72:22-8, 1968; Rauh et al., Am. J. Dis. Child 109:232-7, 1965). Retrospective analysis showed that formalin inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting antibodies, but it did not destroy the ability of the HA (hemagglutinin or attachment) protein to induce neutralizing antibodies (Norrby et al., J. Infect. Dis. 132:262-9, 1975; Norrby et al., Infect. Immun. 11:231-9, 1975). When the immunity induced by the HA protein had waned sufficiently to permit extensive infection with wild type measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication (Bellanti, Pediatrics 48:715-29, 1971; Buser, N. Engl. J. Med. 277:250-1, 1967). This atypical disease is believed to be mediated in part by an altered cell-mediated immune response in which Th-2 cells were preferentially induced leading to heightened disease manifestations at the sites of viral replication (Polack et al., Nat. Med. 5:629-34, 1999). Because of this experience with nonliving measles virus vaccines and also because the immunogenicity of such parenterally-administered vaccines can be decreased by

passively-transferred antibodies, there has been considerable reluctance to evaluate such vaccines in human infants. It should be noted that disease potentiation appears to be associated only with killed vaccines.

Yet another strategy that has been explored for developing a vaccine against measles for use in young infants has been the use of viral vectors to express a protective antigen of the measles virus (Drillien et al., Proc. Natl. Acad. Sci. U.S.A. **85**:1252-6, 1988; Fooks et al., J. Gen. Virol. **79**:1027-31, 1998; Schnell et al., Proc. Natl. Acad. Sci. U.S.A. **93**:11359-65, 1996a; Taylor et al., Virology **187**:321-8, 1992; Wild et al., Vaccine **8**:441-2, 1990; Wild et al., J. Gen. Virol. **73**:359-67, 1992). A variety of vectors have been explored including pox viruses such as the replication-competent vaccinia virus or the replication-defective modified vaccinia virus Ankara (MVA) strain. Replication-competent vaccinia recombinants expressing the F or HA glycoprotein of measles virus were efficacious in immunologically naive vaccinees. However, when they were administered parenterally in the presence of passive antibody against measles virus, their immunogenicity and protective efficacy was largely abrogated (Galletti et al., Vaccine **13**:197-201, 1995; Osterhaus et al., Vaccine **16**:1479-81, 1998; Siegrist et al., Vaccine **16**:1409-14, 1998; Siegrist et al., Dev. Biol. Stand. **95**:133-9, 1998).

Replication-competent vaccinia recombinants expressing the protective antigens of RSV have also been shown to be ineffective in inducing a protective immune response when they are administered parenterally in the presence of passive antibody (Murphy et al., J. Virol. **62**:3907-10, 1988a), but they readily protected such hosts when administered intranasally. Unfortunately, replication-competent vaccinia virus recombinants are not sufficiently attenuated for use in immunocompromised hosts such as persons with human immunodeficiency virus (HIV) infection (Fenner et al., World Health Organization, Geneva, 1988; Redfield et al., N. Engl. J. Med. **316**:673-676, 1987), and their administration by the intranasal route even to immunocompetent individuals would be problematic. Therefore they are not being pursued as vectors for use in human infants, some of whom could be infected with HIV.

The MVA vector, which was derived by more than 500 passages in chick embryo cells (Mayr et al., Infection **3**:6-14, 1975; Meyer et al., J. Gen. Virol. **72**:1031-1038, 1991), has also been evaluated as a potential vaccine vector for the protective antigens of several paramyxoviruses (Durbin et al., J. Infect. Dis. **179**:1345-51, 1999a; Wyatt et al.,

Vaccine 14:1451-1458, 1996). MVA is a highly attenuated host range mutant that replicates well in avian cells but not in most mammalian cells, including those obtained from monkeys and humans (Blanchard et al., J. Gen. Virol. 79:1159-1167, 1998; Carroll et al., Virology

238:198-211, 1997; Drexler et al., J. Gen. Virol. 79:347-352, 1998; Sutter et al., Proc. Natl.

5 Acad. Sci. U.S. A. 89:10847-10851, 1992). Avipox vaccine vectors, which have a host range restriction similar to that of MVA, also have been constructed that express measles virus protective antigens (Taylor et al., Virology 187:321-8, 1992). MVA is non-pathogenic in immunocompromised hosts and has been administered to large numbers of humans without incident (Mayr et al., Zentralbl. Bakteriол. [B] 167:375-90, 1978; Stickle et al.,

10 Dtsch. Med. Wochenschr. 99:2386-92, 1974; Werner et al., Archives of Virology 64:247-256, 1980). Unfortunately, both the immunogenicity and efficacy of MVA expressing a paramyxovirus protective antigen were abrogated in passively-immunized rhesus monkeys

whether delivered by a parenteral or a topical route (Durbin et al., Virology 235:323-332, 1999). The immunogenicity of DNA vaccines expressing measles virus protective antigens

15 delivered parenterally was also decreased in passively-immunized hosts (Siegrist et al., Dev. Biol. Stand. 95:133-9, 1998). Replication-defective vectors expressing measles virus protective antigens are presently being evaluated, including adenovirus-measles virus HA recombinants (Fooks et al., J. Gen. Virol. 79:1027-31, 1998). In this context, MVA

20 recombinants expressing parainfluenza virus antigens, unlike replication-competent vaccinia virus recombinants, lacked protective efficacy when given by a mucosal route to animals with passively-acquired antibodies, and it is unlikely that they, or the similar avipox vectors, can be used in infants with maternally-acquired measles virus antibodies.

Based on the reports summarized above, it appears unlikely that a replication-competent or replication-defective poxvirus vector, or a DNA vaccine, expressing a measles

25 virus protective antigen will be satisfactorily immunogenic or efficacious in infants possessing passively-acquired maternal measles virus-specific antibodies.

A recently developed replication-competent virus vector expressing measles virus HA that replicates in the respiratory tract of animal hosts has been developed, namely, vesicular stomatitis virus (VSV), a rhabdovirus which naturally infects cattle but not humans

30 (Roberts et al., J. Virol. 73:3723-32, 1999; Schnell et al., Proc. Natl. Acad. Sci. U.S.A. 93:11359-65, 1996a). Since VSV is an animal virus that can cause disease in humans,

development of this recombinant for use in humans will require that a VSV backbone that is

satisfactorily attenuated in human infants be first identified (Roberts et al., *J. Virol.* 73:3723-32, 1999), but such clinical studies have not been initiated.

Although there have been numerous advances toward development of effective vaccine agents against PIV and other pathogens, including measles, there remains a clear need in the art for additional tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to these pathogens, particularly among young infants. Among the remaining challenges in this context is the need for additional tools to generate suitably attenuated, immunogenic and genetically stable vaccine candidates for use in diverse clinical settings against one or more pathogens. To facilitate these goals, existing methods for identifying and incorporating attenuating mutations into recombinant vaccine strains and for developing vector-based vaccines and immunization methods must be expanded. Surprisingly, the present invention fulfills these needs and provides additional advantages as described herein below.

SUMMARY OF THE INVENTION

The present invention provides chimeric parainfluenza viruses (PIVs) that are infectious in humans and other mammals and are useful in various compositions to generate desired immune responses against one or more PIVs, or against a PIV and one or more additional pathogens in a host susceptible to infection therefrom. In preferred aspects, the invention provides novel methods for designing and producing attenuated, chimeric PIVs that are useful as vaccine agents for preventing and/or treating infection and related disease symptoms attributable to PIV and one or more additional pathogens. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a chimeric PIV genome or antigenome including a partial or complete PIV vector genome or antigenome combined or integrated with one or more heterologous genes or genome segments that encode single or multiple antigenic determinants of a heterologous pathogen or of multiple heterologous pathogens. Also provided within the invention are methods and compositions incorporating a chimeric PIV for prophylaxis and treatment of infection by both a selected PIV and one or more heterologous pathogens, e.g., a heterologous PIV or a non-PIV pathogen such as a measles virus.

antigenome combined with one or more heterologous gene(s) or genome segment(s) from a second human PIV or a non-PIV pathogen such as measles virus.

The PIV "vector" genome or antigenome typically acts as a recipient or carrier to which are added or incorporated one or more "donor" genes or genome segments of a heterologous pathogen. Typically, polynucleotides encoding one or more antigenic determinants of the heterologous pathogen are added to or substituted within the vector genome or antigenome to yield a chimeric PIV that thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus may exhibit other novel phenotypic characteristics compared to one or both of the vector PIV and heterologous pathogens. For example, addition or substitution of heterologous genes or genome segments within a vector PIV strain may additionally, or independently, result in an increase in attenuation, growth changes, or other desired phenotypic changes as compared with a corresponding phenotype of the unmodified vector virus and/or donor. In one aspect of the invention, chimeric PIVs are attenuated for greater efficacy as a vaccine candidate by incorporation of large polynucleotide inserts which specify the level of attenuation in the resulting chimeric virus dependent upon the size of the insert.

Preferred chimeric PIV vaccine candidates of the invention bear one or more major antigenic determinants of a human PIV, e.g., of HPIV1, HPIV2 or HPIV3, and thus elicit an effective immune response against the selected PIV in human hosts. The antigenic determinant which is specific for a selected human PIV may be encoded by the vector genome or antigenome, or may be inserted within or joined to the PIV vector genome or antigenome as a heterologous polynucleotide sequence from a different PIV. The major protective antigens of human PIVs are their HN and F glycoproteins, although other proteins can also contribute to a protective or therapeutic immune response. In this context, both humoral and cell mediated immune responses are advantageously elicited by representative vaccine candidates within the invention. Thus, polynucleotides encoding antigenic determinants that may be present in the vector genome or antigenome, or integrated therewith as a heterologous gene or genome segment, may encode one or more PIV N, P, C, D, V, M, F, HN and/or L protein(s) or selected immunogenic fragment(s) or epitope(s) thereof from any human PIV.

In addition to having one or more major antigenic determinants of a selected human PIV, preferred chimeric PIV vaccine viruses of the invention bear one or more major antigenic determinants of a second human PIV or of a non-PIV pathogen. In exemplary aspects, the chimeric PIV includes a vector genome or antigenome that is a partial or complete human PIV (HPIV) genome or antigenome, for example of HPIV3, and further includes one or more heterologous gene(s) or genome segment(s) encoding antigenic determinant(s) of at least one heterologous PIV, for example HPIV1 and/or HPIV2. Preferably, the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more heterologous HPIV(s). In alternative embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV1 may be added to or substituted within the partial or complete HPIV3 genome or antigenome. Preferably, the antigenic determinant(s) of HPIV1 is/are selected from HPIV1 HN and F glycoproteins or comprise one or more antigenic domains, fragments or epitopes of the HN and/or F glycoproteins. In various exemplary embodiments, both of the HPIV1 genes encoding the HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes in the HPIV3 vector genome or antigenome. These constructs yield chimeric PIVs that elicit a mono- or poly-specific immune response in humans to HPIV3 and/or HPIV1.

In additional exemplary embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV2 is/are added to, or incorporated within, a partial or complete HPIV3 genome or antigenome, yielding a new or additional immunospecificity of the resultant chimera against HPIV2 alone, or against HPIV3 and HPIV2. In more detailed aspects, one or more HPIV2 genes or genome segments encoding one or more HN and/or F glycoproteins or antigenic domains, fragments or epitopes thereof is/are added to or incorporated within the partial or complete HPIV3 vector genome or antigenome.

In yet additional aspects of the invention, multiple heterologous genes or genome segments encoding antigenic determinants of multiple heterologous PIVs are added to or incorporated within a partial or complete PIV vector genome or antigenome, preferably an HPIV vector genome or antigenome. In one preferred embodiment, heterologous genes or genome segments encoding antigenic determinants from both HPIV1 and HPIV2 are added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. In more detailed aspects, one or more HPIV1 genes or genome segments encoding one or

more HN and/or F glycoproteins (or antigenic domains, fragments or epitopes thereof) and one or more HPIV2 genes or genome segments encoding HN and/or F glycoproteins, antigenic domains, fragments or epitopes, is/are added to or incorporated within the partial or complete HPIV3 vector genome or antigenome. In one example, both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome, which is further modified by addition or incorporation of one or more genes or gene segments encoding single or multiple antigenic determinants of HPIV2. This is readily achieved within the invention, for example, by adding or substituting a transcription unit comprising an open reading frame (ORF) of an HPIV2 HN within the chimeric HPIV3-1 vector genome or antigenome. Following this method, specific constructs exemplifying the invention are provided which yield chimeric PIVs having antigenic determinants of both HPIV1 and HPIV2, as exemplified by the vaccine candidates rPIV3-1.2HN and rPIV3-1 $_{cp45}$.2HN described herein below.

In alternative aspects of the invention, chimeric PIVs of the invention are based on a human PIV vector genome or antigenome which is employed as a recipient for incorporation of major antigenic determinants from a non-PIV pathogen. Pathogens from which one or more antigenic determinants may be adopted into the chimeric PIV vaccine candidate include, but are not limited to, measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses. This assemblage of pathogens that may be thus targeted for vaccine development according to the methods of the invention is exemplary only, and those skilled in the art will understand that the use of PIV vectors for carrying antigenic determinants extends broadly to a large host of additional pathogens.

This, in various alternative aspects of the invention, a human PIV genome or antigenome can be employed as a vector for incorporation of one or more major antigenic determinants from a wide range of non-PIV pathogens. Representative major antigens that can be incorporated within chimeric PIVs of the invention include, but are not limited to the measles virus HA and F proteins; the F, G, SH and M2 proteins of subgroup A and subgroup B respiratory syncytial virus, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus

and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G Protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E protein.

Various human PIV vectors can be employed to carry heterologous antigenic determinants of non-PIV pathogens to elicit one or more specific humoral or cell mediated immune responses against the antigenic determinant(s) carried by the chimeric vaccine virus and hence elicit an effective immune response against the wild-type "donor" pathogen in susceptible hosts. In preferred embodiments, one or more heterologous genes or genome segments from the donor pathogen is joined to or inserted within a partial or complete HPIV3 genome or antigenome. Alternatively, the heterologous gene or genome segment may be incorporated within a chimeric HPIV vector genome or antigenome, for example a partial or complete HPIV3 genome or antigenome bearing one or more genes or genome segments of a heterologous PIV. For example, the gene(s) or genome segment(s) encoding the antigenic determinant(s) of a non-PIV pathogen may be combined with a partial or complete chimeric HPIV3-1 vector genome or antigenome, e.g., as described above having one or both HPIV1 genes encoding HN and F glycoproteins substituted for counterpart HPIV3 HN and F genes. Alternatively, the gene(s) or genome segment(s) encoding the antigenic determinant(s) of a non-PIV pathogen may be combined with a partial or complete chimeric genome or antigenome that incorporates single or multiple antigenic determinants of HPIV2, e.g., an HPIV2 HN gene, within an HPIV1 or HPIV3 vector genome or antigenome, or a chimeric HPIV3-1 vector genome or antigenome as described above. The heterologous gene(s) or genome segment(s) encoding one or more measles antigenic determinant(s) may be combined with any of the PIV vectors or chimeric PIV vectors disclosed herein. In the examples provided herein, the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome, or a chimeric HPIV genome or antigenome comprising a partial or complete HPIV3 genome or antigenome having one or more genes or genome segments encoding antigenic determinant(s) of a heterologous HPIV added or incorporated therein. In one such chimeric construct, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to a HPIV3 vector genome or antigenome at various positions, yielding exemplary chimeric PIV/measles vaccine candidates rPIV3(HA HN-L), rPIV3(HA N-P), *rcp45L*(HA N-P), rPIV3(HA P-M), or *rcp45L*(HA P-M).

In additional exemplary embodiments, the PIV vector genome or antigenome is a chimeric HPIV genome or antigenome comprising a partial or complete HPIV3 genome or antigenome having one or more gene(s) or genome segment(s) encoding one or more antigenic determinant(s) of HPIV1 added or incorporated therein. This construct may be used as a vector, e.g., for measles virus, wherein the heterologous antigenic determinant(s) is/are selected from the measles virus HA and F proteins and antigenic domains, fragments and epitopes thereof. In one example, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to or incorporated within a HPIV3-1 vector genome or antigenome having both the HPIV3 HN and F ORFs substituted by the HN and F ORFs of HPIV1. Among this category of recombinants are vaccine candidates identified herein below as rPIV3-1 HAP-M or rPIV3-1 HAP-M cp45L.

In other detailed embodiments of the invention, the partial or complete PIV vector genome or antigenome is combined with one or more “supernumerary” (i.e., additional to a full complement of genes, whether present in a wild-type vector or in a mutant, e.g., chimeric vector backbone) heterologous gene(s) or genome segment(s) to form the chimeric PIV genome or antigenome. The vector genome or antigenome is often a complete HPIV3 or HPIV3-1 chimeric genome or antigenome, and the supernumerary heterologous gene(s) or genome segment(s) are selected from HPIV1 HN, HPIV1 F, HPIV2 HN, HPIV2 F, measles HA, and/or a translationally silent synthetic gene unit. In certain exemplary embodiments, one or both of the HPIV1 HN and/or HPIV2 HN ORF(s) is/are inserted within the HPIV3 vector genome or antigenome, respectively. In more detailed embodiments, the HPIV1 HN, HPIV2 HN, and measles virus HA ORFs are inserted between the N/P, P/M, and HN/L genes, respectively. Alternatively, the HPIV1 HN and HPIV2 HN genes may be inserted between the N/P and P/M genes, respectively and a 3918-nt GU insert is added between the HN and L genes. Among this category of recombinants are vaccine candidates identified herein below as rHPIV3 1HNN-P, rHPIV3 1HNP-M, rHPIV3 2HNN-P, rHPIV3 2HNP-M, rHPIV3 1HNN-P 2HNP-M, rHPIV3 1HNN-P 2HNP-M HAHN-L, and rHPIV3 1HNN-P 2HNP-M 3918GUHN-L.

Thus designed and constructed, chimeric PIV of the invention may contain protective antigens from one, two, three, four or more different pathogens. For example, vaccine candidates are provided which contain protective antigens from one to four pathogens selected from HPIV3, HPIV1, HPIV2, and measles virus. To construct such multi-specific vaccine candidates, one or more supernumerary heterologous gene(s) or

genome segment(s) can be added which may add a total length of supernumerary foreign sequence to the recombinant genome or antigenome of 30% to 50% or greater (e.g., compared to the wild-type HPIV3 genome length of 15,462 nt). The addition of one or more supernumerary heterologous gene(s) or genome segment(s) in this context often specifies an attenuation phenotype of the chimeric PIV, which exhibits at least a 10-to 100-fold, often 100- to 1,000-fold, and up to a 1,000- to 10,000-fold or greater decrease in replication in the upper and/or lower respiratory tract.

To construct chimeric PIV clones of the invention, a heterologous gene or genome segment of a donor PIV or non-PIV pathogen may be added or substituted at any operable position in the vector genome or antigenome. Often, the position of a gene or gene segment substitution will correspond to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete PIV vector genome or antigenome. In other embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the background genome or antigenome, to enhance or reduce expression, respectively, of the heterologous gene or genome segment. In more detailed aspects of the invention, a heterologous genome segment, for example a genome segment encoding an immunogenic ectodomain of a heterologous PIV or non-PIV pathogen, can be substituted for a corresponding genome segment in a counterpart gene in the PIV vector genome or antigenome to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV or non-PIV pathogen. In alternate embodiments, a chimeric PIV genome or antigenome may be engineered to encode a polyspecific chimeric glycoprotein in the recombinant virus or subviral particle having immunogenic glycoprotein domains or epitopes from two different pathogens. In yet additional embodiments, heterologous genes or genome segments from one PIV or non-PIV pathogen can be added (i.e., without substitution) within a PIV vector genome or antigenome to create novel immunogenic properties within the resultant clone. In these cases, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment, optionally for the additional purpose of attenuating the resultant chimeric virus, in combination with a complete PIV vector genome or antigenome. Alternatively, the heterologous gene or genome segment may be added in conjunction with deletion of a selected gene or genome segment in the vector genome or antigenome.

In preferred embodiments of the invention, the heterologous gene or genome segment is added at an intergenic position within the partial or complete PIV vector genome or antigenome. Alternatively, the gene or genome segment can be inserted within other noncoding regions of the genome, for example, within 5' or 3' noncoding regions or in other positions where noncoding nucleotides occur within the vector genome or antigenome. In some instances, it may be desired to insert the heterologous gene or genome segment at a non-coding site corresponding to or overlapping a cis-acting regulatory sequence within the vector genome or antigenome, e.g., within a sequence required for efficient replication, transcription, and/or translation. These regions of the vector genome or antigenome represent target sites for disruption or modification of regulatory functions associated with introduction of the heterologous gene or genome segment.

For the preferred purpose of constructing candidate vaccine viruses for clinical use, it is often desirable to adjust the attenuation phenotype of chimeric PIV of the invention by introducing additional mutations that increase or decrease the level of attenuation in the recombinant virus. Therefore, in additional aspects of the invention, attenuated, chimeric PIVs are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations that specify an attenuating phenotype in the resultant virus or subviral particle. These attenuating mutations may be generated *de novo* and tested for attenuating effects according to well known rational design mutagenesis strategies. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant PIV or other viruses and thereafter incorporated into a chimeric PIV of the invention.

Preferred attenuating mutations in the latter context are readily identified and incorporated into a chimeric PIV, either by inserting the mutation within the vector genome or antigenome by cloning or mutagenizing the vector genome or antigenome to contain the attenuating mutation. Preferably, attenuating mutations are engineered within the vector genome or antigenome and are imported or copied from biologically derived, attenuated PIV mutants. These are recognized to include, for example, cold passaged (*cp*), cold adapted (*ca*), host range restricted (*hr*), small plaque (*sp*), and/or temperature sensitive (*ts*) PIV mutants. In exemplary embodiments, one or more attenuating mutations present in the well characterized JS HPIV3 *cp*45 mutant strain are incorporated within chimeric PIV of the invention, preferably including one or more mutations identified in the polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS. Alternatively or

additionally, attenuating mutations present in the JS HPIV3 *cp45* mutant strain are introduced in the N protein of chimeric PIV clones, for example which encode amino acid substitution(s) at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS. Yet additional useful attenuating mutations encode amino acid substitution(s) in the C protein, e.g., at a position corresponding to Ile₉₆ of JS and in the M protein, e.g., at a position corresponding to Pro₁₉₉ (for example a Pro₁₉₉ to Thr mutation). Other mutations identified in PIV3 JS *cp45* that can be adopted to adjust attenuation of a chimeric PIV of the invention are found in the F protein, e.g., at a position corresponding to Ile₄₂₀ or Ala₄₅₀ of JS, and in the HN protein, e.g., at a position corresponding to residue Val₃₈₄ of JS.

Attenuating mutations from biologically derived PIV mutants for incorporation into chimeric PIV of the invention also include mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS *cp45*. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS *cp45*.

From PIV3 JS *cp45* and other biologically derived PIV mutants, a large "menu" of attenuating mutations is provided, each of which mutations can be combined with any other mutation(s) for finely adjusting the level of attenuation in chimeric PIV vaccine candidates of the invention. In exemplary embodiments, chimeric PIVs are constructed which include one or more, and preferably two or more, mutations of HPIV3 JS *cp45*. Thus, chimeric PIVs of the invention selected for vaccine use often have two and sometimes three or more attenuating mutations from biologically derived PIV mutants or like model sources to achieve a satisfactory level of attenuation for broad clinical use. Preferably, these attenuating mutations incorporated within recombinant chimeric PIVs of the invention are stabilized by multiple nucleotide substitutions in a codon specifying the mutation.

Introduction of attenuating and other desired phenotype-specifying mutations into a selected PIV vector, including chimeric bovine-human PIV vectors, may be achieved by transferring a heterologous gene or genome segment containing the mutation, e.g., a gene encoding a mutant L protein, or portion thereof, into the PIV vector genome or antigenome. Alternatively, the mutation may be present in the selected vector genome or antigenome, and

the introduced heterologous gene or genome segment may bear no mutations, or may bear one or more additional different mutations.

In certain examples, the vector genome or antigenome is modified at one or more sites corresponding to a site of mutation in a heterologous "donor" virus (e.g., a heterologous bovine or human PIV or a non-PIV negative stranded RNA virus) to contain or encode the same, or a conservatively related, mutation (e.g., a conservative amino acid substitution) as a mutation identified in the donor virus (see, PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference). In one exemplary embodiment, a PIV vector genome or antigenome is modified at one or more sites corresponding to a site of mutation in HPIV3 JS *cp45*, as enumerated above, to contain or encode the same or a conservatively related mutation as that identified in the *cp45* "donor."

Preferred mutant PIV strains for identifying and incorporating attenuating mutations into PIV vectors of the invention include cold passaged (cp), cold adapted (ca), host range restricted (hr), small plaque (sp), and/or temperature sensitive (ts) mutants, for example the JS HPIV3 *cp45* mutant strain. Attenuating mutations from biologically derived PIV mutants for incorporation into human-bovine chimeric PIV of the invention also include mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS *cp45*. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS *cp45*.

Additional mutations which can be adopted or transferred to PIV vectors of the invention may be identified in non-PIV nonsegmented negative stranded RNA viruses and incorporated in PIV mutants of the invention. This is readily accomplished by mapping the mutation identified in a heterologous negative stranded RNA virus to a corresponding, homologous site in a recipient PIV genome or antigenome and mutating the existing sequence in the recipient to the mutant genotype (either by an identical or conservative mutation), as described in PCT/US00/09695 filed April 12, 2000 and its priority U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999, incorporated herein by reference. In accordance with this disclosure, additional attenuating mutations can

be readily adopted or engineered within chimeric PIVs of the invention that are identified in other viruses, particularly other nonsegmented negative stranded RNA viruses.

In yet additional aspects of the invention, chimeric PIVs, with or without attenuating mutations modeled after biologically derived attenuated mutant viruses, are constructed to have additional nucleotide modification(s) to yield a desired phenotypic, structural, or functional change. Typically, the selected nucleotide modification will be made within the partial or complete PIV vector genome, but such modifications can be made as well within any heterologous gene or genome segment that contributes to the chimeric clone. These modifications preferably specify a desired phenotypic change, for example a change in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host range restriction, or immunogenicity. Structural changes in this context include introduction or ablation of restriction sites into PIV encoding cDNAs for ease of manipulation and identification.

In preferred embodiments, nucleotide changes within the genome or antigenome of a chimeric PIV include modification of a viral gene by partial or complete deletion of the gene or reduction or ablation (knock-out) of its expression. Target genes for mutation in this context include any of the PIV genes, including the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F, and the products of the C, D and V open reading frames (ORFs). To the extent that the recombinant virus remains viable and infectious, each of these proteins can be selectively deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to achieve novel deletion or knock out mutants. For example, one or more of the C, D, and/or V genes may be deleted in whole or in part, or its expression reduced or ablated (e.g., by introduction of a stop codon, by a mutation in an RNA editing site, by a mutation that alters the amino acid specified by an initiation codon, or by a frame shift mutation in the targeted ORF(s)). In one embodiment, a mutation can be made in the editing site that prevents editing and ablates expression of proteins whose mRNA is generated by RNA editing (Kato et al., EMBO 16:578-587, 1997 and Schneider et al., Virology 227:314-322, 1997, incorporated herein by reference). Alternatively, one or more of the C, D, and/or V ORF(s) can be deleted in whole or in part to alter the phenotype of the resultant recombinant clone to improve growth, attenuation, immunogenicity or other desired phenotypic characteristics (see, U.S. Patent

Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference).

Alternative nucleotide modifications in chimeric PIV of the invention include a deletion, insertion, addition or rearrangement of a cis-acting regulatory sequence for a selected gene in the recombinant genome or antigenome. In one example, a cis-acting regulatory sequence of one PIV gene is changed to correspond to a heterologous regulatory sequence, which may be a counterpart cis-acting regulatory sequence of the same gene in a different PIV, or a cis-acting regulatory sequence of a different PIV gene. For example, a gene end signal may be modified by conversion or substitution to a gene end signal of a different gene in the same PIV strain. In other embodiments, the nucleotide modification may comprise an insertion, deletion, substitution, or rearrangement of a translational start site within the recombinant genome or antigenome, e.g., to ablate an alternative translational start site for a selected form of a protein.

In addition, a variety of other genetic alterations can be produced in a chimeric PIV genome or antigenome, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV. For example, genes or genome segments from non-PIV sources may be inserted in whole or in part. In one such aspect, the invention provides methods for attenuating chimeric PIV vaccine candidates based on host range effects due to the introduction of one or more gene(s) or genome segment(s) from, e.g., a non-human PIV into a human PIV vector-based chimeric virus. For example, host range attenuation can be conferred on a HPIV-vector based chimeric construct by introduction of nucleotide sequences from a bovine PIV (BPIV) (see, e.g., as disclosed in U.S. Application Serial No. 09/586,479, filed June 1, 2000, corresponding to U.S. Provisional Application Serial No. 60/143,134 filed on July 9, 1999, incorporated herein by reference). These effects are attributed to structural and functional divergence between the vector and donor viruses and provide a stable basis for attenuation. For example, between HPIV3 and BPIV3 the percent amino acid identity for each of the N proteins is 86%, for P is 65%, M 93%, F 83%, HN 77%, and L 91%. All of these proteins are therefore candidates for introduction into a HPIV vector to yield an attenuated chimeric virus which cannot readily be altered by reversion. In exemplary embodiments, the vector genome or antigenome is an HPIV3 genome or antigenome and the heterologous gene or genome segment is a N ORF derived from a selected BPIV3 strain.

or more immunogenic domain(s), fragment(s) or epitope(s) thereof, and the vector is further modified by incorporation of one or more HPIV2 HN and/or F gene(s) or genome segment(s) encoding one or more immunogenic domain(s), fragment(s) or epitope(s) thereof to form the chimeric genome or antigenome which expresses protective antigen(s) from both HPIV1 and HPIV2. This category of chimeric PIV is exemplified by various vaccine candidates identified below as rB/HPIV3.1-2F; rB/HPIV3.1-2HN; or rB/HPIV3.1-2F,2HN.

In yet additional aspects of the invention, the order of genes can be changed to cause attenuation or reduce or enhance expression of a particular gene. Alternatively, a PIV genome promoter can be replaced with its antigenome counterpart to yield additional desired phenotypic changes. Different or additional modifications in the recombinant genome or antigenome can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

In yet additional aspects, polynucleotide molecules or vectors encoding the chimeric PIV genome or antigenome can be modified to encode non-PIV sequences, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein or immunogenic epitope of a microbial pathogen (e.g., virus, bacterium or fungus) capable of eliciting a protective immune response in an intended host. In one such embodiment, chimeric PIVs are constructed that incorporate a gene encoding a cytokine to yield novel phenotypic and immunogenic effects in the resulting chimera.

In addition to providing chimeric PIV for vaccine use, the invention provides related cDNA clones and vectors which incorporate a PIV vector genome or antigenome and heterologous polynucleotide(s) encoding one or more heterologous antigenic determinants, wherein the clones and vectors optionally incorporate mutations and related modifications specifying one or more attenuating mutations or other phenotypic changes as described above. Heterologous sequences encoding antigenic determinants and/or specifying desired phenotypic changes are introduced in selected combinations, e.g., into an isolated polynucleotide which is a recombinant cDNA vector genome or antigenome, to produce a suitably attenuated, infectious virus or subviral particle in accordance with the methods described herein. These methods, coupled with routine phenotypic evaluation, provide a large assemblage of chimeric PIVs having such desired characteristics as attenuation, temperature sensitivity, altered immunogenicity, cold-adaptation, small plaque size, host

isolated polynucleotide molecule encoding a chimeric PIV, and an expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins of a PIV. Upon expression, the genome or antigenome and N, P, and L proteins combine to produce an infectious PIV particle, such as a viral or subviral particle.

5 The chimeric PIVs of the invention are useful in various compositions to generate a desired immune response against one or more PIVs, or against PIV and a non-PIV pathogen, in a host susceptible to infection therefrom. Chimeric PIV recombinants are capable of eliciting a mono- or poly-specific protective immune response in an infected mammalian host, yet are sufficiently attenuated so as to not cause unacceptable symptoms of
10 disease in the immunized host. The attenuated virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

 The invention further provides novel vaccines comprising a physiologically
15 acceptable carrier and/or adjuvant and an isolated attenuated chimeric parainfluenza virus or subviral particle as described above. In preferred embodiments, the vaccine is comprised of a chimeric PIV having at least one, and preferably two or more additional mutations or other nucleotide modifications that specify a suitable balance of attenuation and immunogenicity. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine
20 may comprise attenuated chimeric PIV that elicits an immune response against a single PIV strain or against multiple PIV strains or groups. In this regard, chimeric PIV can be combined in vaccine formulations with other PIV vaccine strains, or with other viral vaccine viruses such as RSV.

 In related aspects, the invention provides a method for stimulating the
25 immune system of an individual to elicit an immune response against one or more PIVs, or against PIV and a non-PIV pathogen, in a mammalian subject. The method comprises administering a formulation of an immunologically sufficient amount a chimeric PIV in a physiologically acceptable carrier and/or adjuvant. In one embodiment, the immunogenic composition is a vaccine comprised of a chimeric PIV having at least one, and preferably
30 two or more attenuating mutations or other nucleotide modifications specifying a desired phenotype and/or level of attenuation as described above. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine may comprise an attenuated

chimeric PIV that elicits an immune response against a single PIV, against multiple PIVs, e.g., HPIV1 and HPIV3, or against one or more PIV(s) and a non-PIV pathogen such as measles or RSV. In this context, chimeric PIVs can elicit a monospecific immune response or a polyspecific immune response against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen. Alternatively, chimeric PIV having different immunogenic characteristics can be combined in a vaccine mixture or administered separately in a coordinated treatment protocol to elicit more effective protection against one PIV, against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen such as measles or RSV. Preferably the immunogenic compositions of the invention are administered to the upper respiratory tract, e.g., by spray, droplet or aerosol. Preferably the immunogenic composition is administered to the upper respiratory tract, e.g., by spray, droplet or aerosol.

The invention also provides novel combinatorial vaccines and coordinate vaccination protocols for multiple pathogenic agents, including multiple PIV's and/or PIV and a non-PIV pathogen. For example, selected targets for early vaccination according to these compositions include RSV and PIV3, which each cause significant amount of illness within the first four months of life, whereas most of the illness caused by PIV1 and PIV2 occurs after six months of age (Collins et al., In Fields Virology, Vol. 1, pp. 1205-1243, Lippincott-Raven Publishers, Philadelphia, 1996; Reed et al., J. Infect. Dis. 175:807-13, 1997). A preferred immunization sequence employing live attenuated RSV and PIV vaccines is to administer RSV and PIV3 as early as one month of age (e.g., at one and two months of age) followed by a bivalent PIV1 and PIV2 vaccine at four and six months of age. It is thus desirable to employ the methods of the invention to administer multiple PIV vaccines, including one or more chimeric PIV vaccines, coordinately, e.g., simultaneously in a mixture or separately in a defined temporal sequence (e.g., in a daily or weekly sequence), wherein each vaccine virus preferably expresses a different heterologous protective antigen. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against each of the three PIV viruses and other pathogens in early infancy.

Importantly, the presence of multiple PIV serotypes and their unique epidemiology with PIV3 disease occurring at an earlier age than that of PIV1 and PIV2 makes it desirable to sequentially immunize an infant with different PIV vectors each expressing the same heterologous antigenic determinant such as the measles virus HA. This

sequential immunization permits the induction of the high titer of antibody to the heterologous protein that is characteristic of the secondary antibody response. In one embodiment, early infants (e.g. 2-4 month old infants) are immunized with an attenuated chimeric virus of the invention, for example a chimeric HPIV3 expressing the measles virus HA protein and also adapted to elicit an immune response against HPIV3, such as rcp45L(HA P-M). Subsequently, e.g., at four months of age the infant is again immunized but with a different, secondary vector construct, such as the rPIV3-1 cp45L virus expressing the measles virus HA gene and the HPIV1 antigenic determinants as the functional, obligate glycoproteins of the vector. Following the first vaccination, the vaccinee will elicit a primary antibody response to both the PIV3 HN and F proteins and to the measles virus HA protein, but not to the PIV1 HN and F protein. Upon secondary immunization with the rPIV3-1 cp45L expressing the measles virus HA, the vaccinee will be readily infected with the vaccine because of the absence of antibody to the PIV1 HN and F proteins and will develop both a primary antibody response to the PIV1 HN and F protective antigens and a high titered secondary antibody response to the heterologous measles virus HA protein. A similar sequential immunization schedule can be developed where immunity is sequentially elicited against HPIV3 and then HPIV2 by one or more of the chimeric vaccine viruses disclosed herein, simultaneous with stimulation of an initial and then secondary, high titer protective response against measles or another non-PIV pathogen. This sequential immunization strategy, preferably employing different serotypes of PIV as primary and secondary vectors, effectively circumvents immunity that is induced to the primary vector, a factor ultimately limiting the usefulness of vectors with only one serotype. The success of sequential immunization with rPIV3 and rPIV3-1 virus vaccine candidates as described above has been demonstrated (Tao et al., Vaccine 17:1100-8, 1999).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate insertion of the HA gene of measles virus into the HPIV3 genome (Note: all of the figures presented herein and related descriptions refer to the positive-sense antigenome of HPIV3, 5' to 3').

Figure 1A provides a diagram (top; not to scale) of the 1926 nt insert containing the complete open reading frame of the hemagglutinin (HA) gene of the Edmonston wildtype strain of measles virus engineered to express the measles virus HA

from an extra transcriptional unit. The insert contains, in 5' to 3' order: an *Afl*II site; nts 3699-3731 from the HPIV3 antigenome which contains the P/M gene junction, including downstream noncoding sequence for the P gene, its gene-end signal, the intergenic region, and the M gene-start signal; three additional nts (GCG); the complete measles virus HA ORF; HPIV3 nt 3594-3623 from the downstream noncoding region of the P gene; and a second *Afl*II site. Figure 1A, Panel 1 illustrates the complete antigenome of the JS wildtype strain of HPIV3 (rPIV3) with the introduced *Afl*II site in the 3'-noncoding region of the N gene before (top) and after (bottom) insertion of the measles HA ORF. Figure 1A, Panel 2 illustrates the complete antigenome of the JS wildtype strain of HPIV3 (rPIV3) with the introduced *Afl*II site in the 3'-noncoding region of the P gene before (top) and after (bottom) insertion of the measles HA ORF. SEQ ID NO. 1 and SEQ ID NO. 2 are shown in Fig. 1A.

Figure 1B provides a diagram (top; not to scale) of the 2028 nt insert containing the complete ORF of the HA gene of measles virus. The insert contains, in 5' to 3' order: a *Stu*I site; nts 8602 to 8620 from the HPIV3 antigenome, which consist of downstream noncoding sequence from the HN gene and its gene-end signal; the conserved HPIV3 intergenic trinucleotide; nts 6733 to 6805 from the HPIV3 antigenome, which contains the HN gene-start and upstream noncoding region; the measles virus HA ORF; HPIV3 nts 8525-8597, which are downstream noncoding sequences from the HN gene; and a second *Stu*I site. The construction is designed to, upon insertion, regenerate the HPIV3 HN gene containing the *Stu*I site, and place the measles virus ORF directly after it flanked by the transcription signals and noncoding region of the HPIV3 HN gene. The complete antigenome of HPIV3 JS wildtype (rPIV3) with the introduced *Stu*I site at nt position 8600 in the 3'-noncoding region of the HN gene is illustrated in the next (middle) diagram. Below is the antigenome of HPIV3 expressing the measles HA protein inserted into the *Stu*I site. The HA cDNA used for this insertion came from an existing plasmid, rather than from the Edmonston wild type measles virus, which was used for the insertions in the N/P and P/M regions. This cDNA had two amino acid differences from the HA protein inserted in Fig 1A, and their location in the HA gene of measles virus is indicated by the asterisks in Figure 1B. SEQ ID NO. 3 and 4 are shown in Fig. 1B.

Figure 2 illustrates expression of the HA protein of measles virus by rHPIV3-measles virus-HA chimeric viruses in LLC-MK2 cells. The figure presents a radioimmunoprecipitation assay (RIPA) demonstrating that the measles HA protein is expressed by the recombinant chimeric viruses *rcp45L*(HA P-M) and *rcp45L*(HA N-P), and

by the Edmonston wild type strain of measles virus (Measles), but not by the rJS wild type HPIV3 (rJS). Lanes A--³⁵S-labeled infected cell lysates were immunoprecipitated by a mixture of three monoclonal antibodies specific to the HPIV3 HN protein). The 64kD band corresponding to the HN protein (open arrow) is present in each of the three HPIV3 infected cell lysates (lanes 3, 5, and 7), but not in the measles virus infected cell lysates (lane 9), confirming that the *rcp45L*(HA P-M) and *rcp45L*(HA N-P) chimeras are indeed HPIV3 and express similar levels of HN proteins. Lanes (b)--³⁵S-labeled infected cell lysates were immunoprecipitated by a mixture of monoclonal antibodies which recognizes the HA glycoprotein of measles virus (79-XV-V17, 80-III-B2, 81-1-366) (Hummel et al., *J. Virol.* 69:1913-6, 1995; Sheshberadaran et al., *Arch. Virol.* 83:251-68, 1985, each incorporated herein by reference). The 76kD band corresponding to the HA protein (closed arrow) is present in lysates from cells infected with the *rcp45L*(HA) chimeric viruses (lanes 6, 8) and the measles virus (lane 10), but not in the lysates from rJS infected cells (lane 4), a HPIV3 wild type virus which does not encode a measles virus HA gene.

Figure 3 illustrates insertion of the HPIV2 HN gene as an extra transcription/translation unit into the antigenomic cDNA encoding rPIV3-1 or rPIV3-1*cp45* chimeric virus (Note: rPIV3-1 is a rPIV3 in which the HN and F genes were replaced by those of HPIV1, and rPIV3-1*cp45* is a version which contains, in addition, 12 mutations from the *cp45* attenuated virus). The HPIV2 HN gene was amplified from vRNA of HPIV2 using RT-PCR with HPIV2 HN gene specific primers (Panel A). The amplified cDNA, carrying a primer-introduced *NcoI* site at its 5'-end and a *HindIII* site at its 3'-end, was digested with *NcoI-HindIII* and ligated into pLit.PIV31HNhc, that had been digested with *NcoI-HindIII*, to generate pLit.PIV32HNhc (Panel B). The pLit.PIV32HNhc plasmid was used as a template to produce a modified PIV2 HN cassette (Panel C), which has a *PpuMI* site at its 5'-end and an introduced *PpuMI* site at its 3'-end. This cassette contained, from left to right: the *PpuMI* site at the 5'-end, a partial 5'-untranslated region (UTR) of PIV3 HN, the PIV2 HN ORF, a 3'-UTR of PIV3 HN, the gene-end, intergenic, gene-start sequence that exists at the PIV3 HN and L gene junction, a portion of the 5'-untranslated region of PIV3 L, and the introduced *PpuMI* site at the 3'-end. This cDNA cassette was digested with *PpuMI* and then ligated to p38'ΔPIV31hc, that had been digested with *PpuMI*, to generate p38'ΔPIV31hc.2HN (Panel D). The 8.5 Kb *BspEI-SphI* fragment was assembled into the *BspEI-SphI* window of pFLC.2G+.hc or pFLC*cp45* to generate the final full-length antigenomic cDNA, pFLC.3-1hc.2HN (Panel E) or pFLC.3-1hc.*cp45*.2HN (Panel F), respectively. pFLC.2G+.hc and pFLC*cp45* are full-length antigenomic clones encoding wild

type rPIV3-1 and rPIV3_{cp45}, respectively, that have been described previously (Skiadopoulos et al., *J. Virol.* **73**:1374-81, 1999a; Tao et al., *J. Virol.* **72**:2955-2961, 1998, incorporated herein by reference).

Figure 4 details and verifies construction of the rPIV3-1.2HN chimeric virus carrying the PIV2 HN ORF insert between the PIV1 F and HN genes. Panel A depicts the differences in the structures of rPIV3-1 and rPIV3-1.2HN, which contains the PIV2 HN ORF insert between the PIV1 F and HN ORFs of rPIV3-1. The arrows indicate the approximate locations of the RT-PCR primers used to amplify fragments analyzed in Panels B-D. Panels B and C depict the expected sizes of the restriction enzyme digestion fragments generated from the RT-PCR products amplified from rPIV3-1 and rPIV3-1.2HN using either the *Ppu*MI or *Nco*I restriction endonucleases, with the fragment sizes in base pairs (bp) indicated, and the results presented in panel D. vRNA extracted from virus harvested from rPIV3-1.2HN or from rPIV3-1 infected LLC-MK2 cells was used as a template in the presence and absence of reverse transcriptase (RT) to amplify cDNA fragments by PCR using primers indicated in panel A. PCR fragments were absent in RT-PCR reactions lacking RT indicating that the template employed for amplification of the DNA fragments was RNA and not contaminating cDNA (Lanes A and C of panel D). When the RT step was included, rPIV3-1.2HN vRNA (Lane B) yielded a fragment that was approximately 2kb larger than that of its rPIV3-1 parent (Lane D) indicating the presence of an insert of 2kb. Furthermore, digestion of this 3kb fragment with several different restriction endonucleases indicated that the RT-PCR fragment from rPIV3-1.2HN (odd numbered lanes) has patterns that are different from those of the rPIV3-1 parent (even numbered lanes) for each restriction endonuclease tested. For each digestion, the number of sites and the sizes of the fragments obtained were completely consistent with the predicted sequence of the RT-PCR products of rPIV3-1 and rPIV3-1.2HN. Representative examples are presented. First, the *Ppu*MI digestion of the RT-PCR product from rPIV3-1.2HN (Lane 1) produced three bands of the expected sizes indicating the presence of two *Ppu*MI sites and *Ppu*MI digestion of the RT-PCR product from rPIV3-1 produced two bands of the expected sizes for rPIV3-1 (Lane 2) indicating the presence of just one *Ppu*MI site. Second, the *Nco*I digestion of the RT-PCR product from rPIV3-1.2HN (Lane 5) produced 4 bands including the 0.5 kb fragment indicative of the HPIV2 HN gene and the *Nco*I digestion of the RT-PCR product from rPIV3-1 (Lane 6) produced the expected two fragments. M identifies the lane containing the 1 kb DNA ladder used as nucleotide (nt) size markers (Life Technology). Similar results confirmed the presence of the HPIV2 HN insert in rPIV3-1_{cp45}.2HN.

Figure 5 demonstrates that rPIV3-1.2HN expresses the HPIV2 HN protein. LLC-MK2 monolayers were infected with rPIV3-1, rPIV3-1.2HN, or the PIV2/V94 wild type virus at a MOI of 5. Infected monolayers were incubated at 32°C and labeled with ³⁵S-met and ³⁵S-cys mixture from 18-36 hours post-infection. Cells were harvested and lysed, and the proteins were immunoprecipitated with anti-HPIV2 HN mAb 150S1 (Durbin et al., *Virology* 261:319-330, 1999; Tsurudome et al., *Virology* 171:38-48, 1989, incorporated herein by reference). Immunoprecipitated samples were denatured, separated on a 4-12% SDS PAGE gel, and autoradiographed (Lanes: 1, rPIV3-1; 2, rPIV3-1.2HN; 3, PIV2/V9412-6). The mAb, specific to HPIV2 HN, precipitated a protein from both rPIV3-1.2HN and PIV2/V94 infected LLC-MK2 cells, but not from rPIV3-1-infected cells, with a size expected for the 86kD Kd HN protein of HPIV2 (Rydbeck et al., *J. Gen. Virol.* 69:931-5, 1988, incorporated herein by reference).

Figure 6 depicts the location and construction of gene unit (GU) insertions or HN gene 3'-noncoding region (NCR) extensions. The nucleotide sequences and unique restriction enzyme cloning sites of the GU and NCR insertion sites are shown in panels A and B, respectively. Cis-acting transcriptional signal sequences, i.e., gene-end (GE), intergenic (IG), and gene-start (GS) signal sequences, are indicated. In Figure 6, Panel A, an oligonucleotide duplex specifying the HN GE, IG and GS signal sequences as well as the unique restriction enzyme recognition sequences are shown inserted into the introduced *StuI* restriction site (underlined nucleotides) (see Figure 1B and Example I for the location of the introduced *StuI* site). A restriction fragment from an RSV antigenome plasmid was cloned into the *HpaI* site. As necessary, a short oligonucleotide duplex was inserted into the *MluI* site of the multiple cloning site, so that the total length of the insert would conform to the rule of six. In Figure 6, Panel B, HN gene 3'-NCR insertions were cloned into the *HpaI* site of the indicated 32 nt multiple cloning site, which had been cloned into the *StuI* restriction site as described in Figure 6, Panel A. Inserted sequences were made to conform to the rule of six by insertion of short oligonucleotide duplexes into the *MluI* site in the multiple cloning site. SEQ ID NO. 5 and 6 are shown in Fig. 6.

Figure 7 illustrates open reading frames (ORFs) in the 3079 bp RSV insert. The six possible reading frames in the 3079 bp RSV fragment are shown (three in each orientation; 3, 2, 1, -1, -2, -3). Short bars represent translation start codons. Long bars represent translation stop codons. The 3079 bp fragment was inserted into the HN 3' NCR (NCR ins) or between the HN and L genes as a gene unit (GU ins) in such an orientation that

the reading frames encountered by the PIV3 translation machinery correspond to -3, -2 and -1 in the figure. These reading frames contain numerous stop codons across the entire length of the sequence, and should therefore not produce any functional proteins.

Figure 8 demonstrates that rPIV3 insertion and extension mutants contain inserts of the appropriate size. RT-PCR was performed using a PIV3-specific primer pair flanking the insertion site, and RT-PCR products were separated by agarose gel electrophoresis. The expected size of the RT-PCR fragment for rPIV3wt (also referred to as rJS) is 3497 bp and that for each of the other rPIV3s GU or NCR mutants is increased in length depending on the size of the insertion. Panel A depicts GU insertion (ins) mutants: 1. rPIV3 wt; 2. r168 nt GU ins; 2. r678 nt GU ins; 3. r996 nt GU ins; 4. r1428 nt GU ins; 5. r1908 nt GU ins; 6. r3918 nt GU ins. M: *Hind*III restriction enzyme digestion products of lamda phage DNA. Sizes of relevant size markers are indicated. Panel B depicts NCR insertion mutants: 1. rPIV3 wt; 2. r258 nt NCR ins; 3. r972 nt NCR ins; 4. r1404 nt NCR ins; 5. r3126 nt NCR ins; 6. r3894 nt NCR ins. M: *Hind*III restriction enzyme digestion products of lambda phage DNA. Sizes of relevant size markers are indicated.

Figures 9A-9C present multi-step growth curves of GU and NCR insertion mutations compared with rHPIV3 wt and rcp45_L. LLC-MK2 monolayers in 6-well plates were infected with each HPIV3 in triplicate at a multiplicity of infection (m.o.i.) of 0.01 and were washed 4 times after removal of the virus supernatant. At 0 hr and at 24 hrs intervals for 6 days post-infection, 0.5 ml virus medium from each well was harvested and 0.5 ml fresh medium was added to each well. Harvested samples were stored at -80°C. Virus present in the samples was quantified by titration on LLC-MK2 monolayers in 96-well plates incubated at 32°C. The titers of viruses are expressed as TCID₅₀/ml. The average of three independent infections from one experiment is shown. The lower limit of detection is 0.7 log₁₀TCID₅₀/ml. Figure 9A-GU insertion mutants; Figure 9B-NCR insertion mutants; Figure 9C-cp45_L/GU insertion mutant.

Figure 10 illustrates the strategy for placing a supernumerary gene insert between the P and M genes of rHPIV3. The downstream (3') NCR of the rHPIV3 P gene was modified to contain an AflII restriction site at antigenomic sequence positions 3693-3698 (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference). This site was then used to insert an oligonucleotide duplex (shown at the top) that contains HPIV3 cis-acting transcriptional signal sequences, i.e., gene-end (GE), intergenic (IG), and gene-start

(GS) motifs. The duplex also contains a series of restriction enzyme recognition sequences available for insertion of foreign ORFs. In the case of the HPIV1 and HPIV2 HN ORFs, the cloning sites were *Nco*I and *Hind*III. Insertion of a foreign ORF into the multiple cloning sites places it under the control of a set of HPIV3 transcription signals, so that in the final recombinant virus the gene is transcribed into a separate mRNA by the HPIV3 polymerase. As necessary, a short oligonucleotide duplex was inserted into the *Mlu*I site of the multiple cloning site to adjust the final length of the genome to be an even multiple of six, which has been shown to be a requirement for efficient RNA replication (Calain et al., *J. Virol.* 67:4822-30, 1993; Durbin et al., *Virology* 234:74-83, 1997b). A similar strategy was used to place HPIV1 and HPIV2 gene inserts between the N and P genes of rHPIV3 using an introduced *Afl*III restriction site at positions 1677-1682 9 (SEQ ID NO. 7).

Figure 11 is a diagram (not to scale) of the genomes of a series of chimeric rHPIV3s that contain one, two or three supernumerary gene inserts, each of which encodes a protective antigen of PIV1, PIV2, or measles virus. Schematic representation of rHPIV3s (not to scale) showing the relative position of the added insert(s) encoding the HN (hemagglutinin-neuraminidase) glycoprotein of HPIV1 (■) or HPIV2 (▤) or the HA (hemagglutinin) glycoprotein of measles virus (▨) inserted into the rHPIV3 backbone (□). The rHPIV3 construct that is diagrammed at the bottom contains a 3918-nt insert (GU) that does not encode a protein (▩) (Skiadopoulos et al., *Virology* 272:225-34, 2000, incorporated herein by reference). Each foreign insert is under the control of a set of HPIV3 gene start and gene end transcription signals and is expressed as a separate mRNA. **a.** LLC-MK2 monolayers on 6 well plates (Costar) were separately infected in triplicate at an m.o.i. of 0.01 with each of the indicated viruses. Supernatants were harvested on days 5, 6 and 7 and virus was quantified as described previously (Skiadopoulos et al., *Virology* 272:225-34, 2000). The mean peak titer obtained for each virus is shown as log₁₀ TCID₅₀/ml. **b.** Mean of two experiments. Serially-diluted viruses were incubated at 32°C and 39°C on LLC-MK2 monolayer cultures for 7 days, and the presence of virus was determined by hemadsorption with guinea pig erythrocytes. The mean reduction in titer at 39°C compared to that of 32°C is shown.

Figure 12 provides a diagram (not to scale) illustrating insertion of a supernumerary gene insert into an rHPIV3 backbone, rHPIV3-N_B, in which the HPIV3 N ORF has been replaced by its BPIV3 counterpart, conferring an attenuation phenotype due to host range restriction (Bailly et al., *J. Virol.* 74:3188-3195, 2000a, incorporated herein by




reference). Schematic representations are shown of rHPIV3 (top) and biologically derived BPIV3 (bottom). The relative position of the N ORF sequence derived from the Kansas strain of BPIV3 () and the measles virus hemagglutinin gene () in the PIV3 backbone are shown. In each case, the foreign sequence is under the control of a set of HPIV3 transcription signals. A portion of the plasmid vector containing the NgoMIV site is shown (). Designations are provided for the antigenomic cDNA clones (left) and their encoded recombinant viruses (right).

Figure 13 illustrates insertion of RSV G or F as an additional, supernumerary gene in a promoter-proximal position into the genome of rB/HPIV3. rB/HPIV3 is a recombinant version of BPIV3 in which the BPIV3 F and HN genes have been replaced by their HPIV3 counterparts (F_H and HN_H respectively). A *B*l*p*I site was created in the B/HPIV3 backbone immediately upstream of the ATG start codon of the N ORF. The RSV G or F open reading frames (ORFs) were inserted into this *B*l*p*I site. The downstream end of either RSV insert was designed to contain a PIV3 gene end (GE) and gene start (GS) sequences (AAGTAAGAAAAA (SEQ ID NO. 8) and AGGATTAAAG, respectively, in positive sense) separated by the intergenic sequence CTT. Each insert also contained an *Nhe*I site that can serve as an insertion site for an additional supernumerary gene.

AGGATTAAAGAACTTTACCGAAAGGTAAGGGGAAAGAAATCCTAAGAGCTTAG
CGATG (SEQ ID NO. 9). GCTTAGCGATG (SEQ ID NO. 10). AAGCTAGCGCTTAGC
(SEQ ID NO. 11). GCTTAGCAAAAAGCTAGCACAATG (SEQ ID NO. 12).

Figure 14 illustrates multicycle replication of rB/HPIV3-G1, rB/HPIV3-F1 and their recombinant and biological parent viruses in simian LLC-MK2 cells. Triplicate monolayer cultures were infected at an input MOI of 0.01 TCID₅₀ per cell with rB/HPIV3-G1, rB/HPIV3-F1, or the following control viruses: rBPIV3 Ka, which is the recombinant version of BPIV3 strain Ka; rB/HPIV3, with is the version of rBPIV3 in which the BPIV3 F and HN glycoprotein genes were replaced with their HPIV3 counterparts; HPIV3 JS, which is biologically-derived HPIV3 strain JS; and BPIV3 Ka, which is the biologically-derived version of BPIV3 strain Ka. The virus titers are shown as mean log₁₀ TCID₅₀/ml of triplicate samples. The lower limit of detection of this assay is 10^{1.45} TCID₅₀/ml.

Figure 15 is a diagram (not to scale) of the genomes of rBPIV3 (#1) and a series of chimeric rB/HPIV3s (#2-6) that contain substitutions of BPIV3 F and HN genes by those of HPIV3 (#2) or HPIV1 (#3-6), and one or two supernumerary gene inserts encoding

the F and/or HN ORF of HPIV2 (#4-6). Schematic representation of the rB/HPIV3.1
chimeric viruses (not to scale) showing the relative position of the supernumerary gene
encoding the F or HN glycoprotein of HPIV2 (F2 and HN2, respectively). Each foreign
insert is under the control of a set of HPIV3 gene start and gene end transcription signals and
5 is designed to be expressed as a separate mRNA.





Figure 16 provides a diagram (not to scale) illustrating the insertion of a the
measles virus HA coding sequence into several different rPIV3 backbones. Three backbones
are illustrated: wild type rHPIV3 (top construct); wild type rHPIV3-1 (second construct from
top) (Tao et al. *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference) in which the
10 HPIV3 F and HN glycoprotein genes have been replaced by those of HPIV1; and rHPIV3-
1cp45L (third construct), a derivative of wild type rHPIV3-1 that contains three attenuating
amino acid point mutations in the L gene derived from the cp45 vaccine strain (Skiadopoulos
et al., *J. Virol.* 72:1762-8, 1998, incorporated herein by reference). The relative position of
the HPIV1 F and HN ORF sequences () and the measles virus HA gene () in the
15 rPIV3 backbone () are shown. In each case, each foreign ORF is under the control of a
set of HPIV3 transcription signals. The relative locations of the three cp45 L amino acid
point mutations in the L gene are indicated (*). A portion of the plasmid vector is containing
the unique NgoMIV site is shown ().

Figure 17 illustrates construction of the PIV3-PIV2 chimeric antigenomic
20 cDNA pFLC.PIV32hc encoding the full-length PIV2 HN and F proteins. The cDNA
fragment containing the full-length PIV2 F ORF flanked by the indicated restriction sites
(A1) was amplified from PIV2/V94 vRNA using RT-PCR and a PIV2 F specific primer pair
(1, 2 in Table 22). This fragment was digested with NcoI plus BamHI (C1) and ligated to the
NcoI-BamHI window of pLit.PIV31.fhc (B1) to generate pLit.PIV32Fhc (D1). In parallel,
25 the cDNA fragment containing the full-length PIV2 HJN ORF flanked by the indicated
restriction sites (A2) was amplified from PIV2/V94 vRNA using RT-PCR and a PIV2 HN
specific primer pair (3, 4 in Table 22). This fragment was digested with NcoI plus HindIII
(C2) and ligated to the NcoI-HindIII window of pLit.PIV31.HNhc (B2) to generate
pLit.PIV32HNhc (D2). pLit.PIV32Fhc and pLit.PIV32HNhc were digested with PpuMI and
30 Spel and assembled together to generate pLit.PIV32hc (E). pLit.PIV32hc was further
digested with BspEI and Spel and introduced into the BspEI-Spel window of p38'ΔPIV31hc

(F) to generate p38' ΔPIC32hc (G). The chimeric PIV3-PIV2 construct was introduced into the BspEI-SphI window of pFLC.2G+hc to generate pFLC.PIC32hc (H).

Figure 18 depicts construction of full-length PIV3-PIV2 chimeric antigenomic cDNA pFLC.PIV32TM and pFLC.PIV32TMcp45, which encode F and HN proteins containing PIV2-derived ectodomains and PIV3-derived transmembrane and cytoplasmic domains. The region of the PIV3 F ORF, in pLit.PIV3.F3a (A1), encoding the ectodomain was deleted (C1) by PCR using a PIV3 F specific primer pair (9, 10 in Table 22. The region of the PIV2 F ORF encoding the ectodomain was amplified from pLit.PIV32Fhc (B1) using PCR and PIV2 F specific primer pair (5, 6 in Table 22). The two resulting fragments (C1 and D1) were ligated to generate pLit.PIV32FTM (E1). In parallel, the region of the PIV3 HN ORF, in pLit.PIV3.HN4 (A2), encoding the ectodomain was deleted (C2) by PCR using a PIV3 HN specific primer pair (11, 12 in Table 22). The region of the PIV2 HN ORF encoding the ectodomain was amplified from pLit.PIV32HNhc (B2) by PCR and a PIV2 HN specific primer pair (8, 9 in Table 22). Those two DNA fragments (C2 and D2) were ligated together to generate pLit.PIV32HNTM (E2). pLit.PIV32FTM and pLit.PIV32HNTM were digested with PpuMI and SpeI and assembled to generate pLit.PIV32TM (F). The BspEI-SpeI fragment from pLit.PIV32TM was ligated to the BspEI-SpeI window of p38' _PIV31hc (G) to generate p38' _PIV32TM (H). The insert containing chimeric PIV3-PIV2 F and HN was introduced as a 6.5 kb BspEI-SphI fragment into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45 to generate pFLC.PIV32TM and pFLC.PIV32TMcp45 (I), respectively.

Figure 19 shows construction of full-length PIV3-PIV2 chimeric antigenomic cDNA pFLC.PIV32CT and pFLC.PIV32Ctcp45 which encode F and HN proteins containing a PIV2-derived ectodomain, a PIV2-derived transmembrane domain, and a PIV3-derived cytoplasmic domain. The region of the PIV3 F ORF in pLit.PIV3.F3a (A1) encoding the ectodomain and the transmembrane domain was deleted (C1) by PCR using a PIV3 F specific primer pair (17, 18 in Table 22). The region of the PIV2 F ORF encoding the ectodomain plus the transmembrane domain was amplified from pLit.PIV32Fhc (B1) using PCR and a PIV2 F specific primer pair (13, 14 in Table 22). The two resulting fragments (C1 and D1) were ligated to generate pLit.PIV32FCT (E1). In parallel, the region of the PIV3 HN ORF in pLit.PIV3.HN4 (A2), encoding the ectodomain and transmembrane domain was deleted (C2) by PCR using a PIV3 HN specific primer pair (19, 20 in Table 22). The region of the PIV2 HN ORF encoding the ectodomain plus the transmembrane domain

was amplified from pLit.PIV32HNhc (B2) by PCR using a PIV2 HN specific primer pair (15, 16 in Table 22). Those two DNA fragments (C2 and D2) were ligated to generate pLit.PIV32HNCT (E2). pLit.PIV32FCT and pLit.PIV32HNCT were digested with PpuMI and SpeI and assembled to generate pLit.PIV32CT (F). The BspEI-SpeI fragment from pLit.PIV32CT was ligated to the BspEI-SpeI window of p38' _PIV31hc (G) to generate p38' _PIV32CT (H). The insert containing chimeric PIV3-PIV2 F and HN was introduced as a 6.5 kb BspEI-SphI fragment into the BspEI-SphI window of pFLC.2G+.hc and pFLC.*cp45* to generate pFLC.PIV32CT and pFLC.PIV32CT*cp45* (I), respectively.

Figure 20 details genetic structures of the PIV3-PIV2 chimeric viruses and the gene junction sequences for rPIV3-2CT and rPIV3-2TM. Panel A illustrates the genetic structures of rPIV3-2 chimeric viruses (middle three diagrams) are compared with that of rPIV3 (top diagram) and rPIV3-1 (bottom diagram) viruses. The *cp45* derivatives are shown marked with arrows depicting the relative positions of *cp45* mutations. For the *cp45* derivatives, only the F and HN genes are different while the remaining genes remained identical, all from PIV3. From top to bottom, the three chimeric PIV3-PIV2 viruses carry decreasing amount of PIV3 glycoprotein genes. Note that rPIV3-2, carrying the complete PIV2 HN and F ORF, was not recoverable. Panel B provides the nucleotide sequences of the junctions of the chimeric F and HN glycoprotein genes for rPIV3-2TM are given along with the protein translation. The shaded portions represent sequences from PIV2. The amino acids are numbered with respect to their positions in the corresponding wild type glycoproteins. Three extra nucleotides were inserted in PIV3-PIV2 HN TM as indicated to make the construct conform to rule of six. Panel C shows the nucleotide sequences of the junctions of the chimeric F and HN glycoprotein genes for rPIV3-2CT, given along with the protein translation. The shaded portions represent sequences from PIV2. The amino acids are numbered with respect to their positions in the corresponding wild type glycoproteins. GE= gene end; I= intergenic; GS= gene start; ORF= open reading frame; TM= transmembrane domain; CT= cytoplasmic domain; *= stop codon.

Figure 21 documents multicycle replication of rPIV3-2 chimeric viruses compared with that of rPIV3/JS and PIV2/V94 wild type parent viruses. Panel A—the rPIV3-2TM and rPIV3-2TM*cp45* viruses, along with the rPIV3/JS and PIV2/V94 wt parent viruses, were used to infect LLC-MK2 cells in 6 well plates, each in triplicate, at an MOI of 0.01. All cultures were incubated at 32°C. After a 1 hour adsorption period, the inocula were removed, and the cells were washed three times with serum-free OptiMEM. The

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cultures were overlaid with 2 ml per well of the same medium. For rPIV3-2TM and rPIV3-2TM_{cp45} infected plates, 0.5 mg/ml of p-trypsin was added to each well. Aliquots of 0.5 ml were taken from each well at 24 hour intervals for 6 days, flash frozen on dry ice, and stored at -80°C. Each aliquot was replaced with 0.5 ml of fresh medium with or without p-trypsin as indicated above. The virus present in the aliquots was titered on LLC-MK2 plates with liquid overlay at 32°C for 7 days, and the endpoints were identified with hemadsorption. Panel B—The rPIV3-2CT and rPIV3-2CT_{cp45}, along with the rPIV3/JS and PIV2/V94 wt parent viruses, were used to infect LLC-MK2 in 6 well plates, each in triplicate, as described in Panel A. Aliquots were taken and processed in the same manner as described in Panel A. Virus titers are expressed as log₁₀TCID₅₀/ml ± standard errors for both experiments presented in Panel A and B.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The instant invention provides methods and compositions for the production and use of novel, chimeric parainfluenza viruses (PIVs) and associated vaccines. The chimeric viruses of the invention are infectious and immunogenic in humans and other mammals and are useful for generating immune responses against one or more PIVs, for example against one or more human PIVs (HPIVs). Alternatively, chimeric PIVs are provided that elicit an immune response against a selected PIV and one or more additional pathogens, for example against both a HPIV and measles virus. The immune response elicited can involve either or both humoral and/or cell mediated responses. Preferably, chimeric PIVs of the invention are attenuated to yield a desired balance of attenuation and immunogenicity for vaccine use.

The invention thus provides novel methods for designing and producing attenuated, chimeric PIVs that are useful as vaccine agents for preventing and/or treating infection and related disease symptoms attributable to PIV and other pathogens. In accordance with the methods of the invention, chimeric parainfluenza viruses or subviral particles are constructed using a PIV “vector” genome or antigenome that is recombinantly modified to incorporate one or more antigenic determinants of a heterologous pathogen. The vector genome or antigenome is comprised of a partial or complete PIV genome or antigenome, which may itself incorporate nucleotide modifications such as attenuating mutations. The vector genome or antigenome is modified to form a chimeric structure

through incorporation of a heterologous gene or genome segment. More specifically, chimeric PIVs of the invention are constructed through a cDNA-based virus recovery system that yields recombinant viruses that incorporate a partial or complete vector or "background" PIV genome or antigenome combined with one or more "donor" nucleotide sequences encoding the heterologous antigenic determinant(s). Preferably the PIV vector comprises a HPIV genome or antigenome, although non-human PIVs, for example a bovine PIV (BPIV), can be employed as a vector to incorporate antigenic determinants of human PIVs and other human pathogens. In exemplary embodiments described herein, a human PIV3 (HPIV3) vector genome or antigenome is modified to incorporate one or more genes or genome segments that encode antigenic determinant(s) of one or more heterologous PIVs (e.g., HPIV1 and/or HPIV2), and/or a non-PIV pathogen (e.g., measles virus). Thus constructed, chimeric PIVs of the invention may elicit an immune response against a specific PIV, e.g., HPIV1, HPIV2, and/or HPIV3, or against a non-PIV pathogen. Alternatively, compositions and methods are provided for eliciting a polyspecific immune response against multiple PIVs, e.g., HPIV1 and HPIV3, or against one or more HPIVs and a non-PIV pathogen such as measles virus.

Exemplary chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome as described above, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components. Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

In preferred aspects of the invention, chimeric PIV incorporate a partial or complete human PIV vector genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a second human PIV or a non-PIV pathogen such as measles virus. The PIV "vector" genome or antigenome typically acts as a recipient or carrier to which are added or incorporated one or more "donor" genes or genome segments of a heterologous pathogen. Typically, polynucleotides encoding one or more antigenic determinants of the heterologous pathogen are added to or substituted within the vector genome or antigenome to yield a chimeric PIV that thus acquires the ability to elicit

an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus may exhibit other novel phenotypic characteristics compared to one or both of the vector PIV and heterologous pathogens.

5 The partial or complete vector genome or antigenome generally acts as a backbone into which heterologous genes or genome segments of a different pathogen are incorporated. Often, the heterologous pathogen is a different PIV from which one or more gene(s) or genome segment(s) is/are of are combined with, or substituted within, the vector genome or antigenome. In addition to providing novel immunogenic characteristics, the addition or substitution of heterologous genes or genome segments within the vector PIV strain may confer an increase or decrease in attenuation, growth changes, or other desired phenotypic changes as compared with the corresponding phenotype(s) of the unmodified vector and donor viruses. Heterologous genes and genome segments from other PIVs that may be selected as inserts or additions within chimeric PIV of the invention include genes or genome segments encoding the PIV N, P, C, D, V, M, F, HN and/or L protein(s) or one or more antigenic determinant(s) thereof.

10 Heterologous genes or genome segments of one PIV may be added as a supernumerary genomic element to a partial or complete genome or antigenome of a different PIV. Alternatively, one or more heterologous gene(s) or genome segment(s) of one PIV may be substituted at a position corresponding to a wild-type gene order position of a counterpart gene(s) or genome segment(s) that is deleted within the PIV vector genome or antigenome. In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promotor-distal compared to a wild-type gene order position of the counterpart gene or genome segment within the vector genome or antigenome to enhance or reduce, respectively, expression of the heterologous gene or genome segment.

15 The introduction of heterologous immunogenic proteins, protein domains and immunogenic epitopes to produce chimeric PIV is particularly useful to generate novel immune responses in an immunized host. Addition or substitution of an immunogenic gene or genome segment from one, donor pathogen within a recipient PIV vector genome or antigenome can generate an immune response directed against the donor pathogen, the PIV vector, or against both the donor pathogen and vector.

To achieve this purpose, chimeric PIV may be constructed that express a chimeric protein, for example an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to a vector fused to a heterologous ectodomain of a different PIV or non-PIV pathogen to provide a fusion protein that elicits an immune response against the heterologous pathogen. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV1 HN or F glycoprotein may be joined with a genome segment encoding the corresponding HPIV3 HN or F glycoprotein cytoplasmic and transmembrane domains to form a HPIV3-1 chimeric glycoprotein that elicits an immune response against HPIV1.

Briefly, PIV of the invention expressing a chimeric glycoprotein comprise a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and a HPIV vector genome or antigenome that is modified to encode a chimeric glycoprotein. The chimeric glycoprotein incorporates one or more heterologous antigenic domains, fragments, or epitopes of a second, antigenically distinct HPIV. Preferably, this is achieved by substitution within the HPIV vector genome or antigenome of one or more heterologous genome segments of the second HPIV that encode one or more antigenic domains, fragments, or epitopes, whereby the genome or antigenome encodes the chimeric glycoprotein that is antigenically distinct from the parent, vector virus.

In more detailed aspects, the heterologous genome segment or segments preferably encode a glycoprotein ectodomain or immunogenic portion or epitope thereof, and optionally include other portions of the heterologous or "donor" glycoprotein, for example both an ectodomain and transmembrane region that are substituted for counterpart glycoprotein ecto- and transmembrane domains in the vector genome or antigenome. Preferred chimeric glycoproteins in this context may be selected from HPIV HN and/or F glycoproteins, and the vector genome or antigenome may be modified to encode multiple chimeric glycoproteins. In preferred embodiments, the HPIV vector genome or antigenome is a partial HPIV3 genome or antigenome and the second, antigenically distinct HPIV is either HPIV1 or HPIV2. In one exemplary embodiment described below, both glycoprotein ectodomain(s) of HPIV2 HN and F glycoproteins are substituted for corresponding HN and F glycoprotein ectodomains in the HPIV3 vector genome or antigenome. In another exemplary embodiment, PIV2 ectodomain and transmembrane regions of one or both HN and/or F glycoproteins are fused to one or more corresponding PIV3 cytoplasmic tail region(s) to form the chimeric glycoprotein. Further details concerning these aspects of the

invention are provided in United States Patent Application entitled CONSTRUCTION AND USE OF RECOMBINANT PARAINFLUENZA VIRUSES EXPRESSING A CHIMERIC GLYCOPROTEIN, filed on December 10, 1999 by Tao et al. and identified by Attorney Docket No. 17634-000340, incorporated herein by reference.

5 To construct chimeric PIVs of the invention carrying a heterologous antigenic determinant of a non-PIV pathogen, a heterologous gene or genome segment of the donor pathogen may be added or substituted at any operable position in the vector genome or antigenome. In one embodiment, heterologous genes or genome segments from a non-PIV pathogen can be added (i.e., without substitution) within a PIV vector genome or antigenome to create novel immunogenic properties within the resultant clone. In these cases, the
10 heterologous gene or genome segment may be added as a supernumerary gene or genome segment, optionally for the additional purpose of attenuating the resultant chimeric virus, in combination with a complete PIV vector genome or antigenome. Alternatively, the heterologous gene or genome segment may be added in conjunction with deletion of a
15 selected gene or genome segment in the vector genome or antigenome.

In preferred embodiments of the invention, the heterologous gene or genome segment is added at an intergenic position within the partial or complete PIV vector genome or antigenome. Alternatively, the gene or genome segment can be inserted within other noncoding regions of the genome, for example, within 5' or 3' noncoding regions or in other
20 positions where noncoding nucleotides occur within the vector genome or antigenome. In one aspect, the heterologous gene or genome segment is inserted at a non-coding site overlapping a cis-acting regulatory sequence within the vector genome or antigenome, e.g., within a sequence required for efficient replication, transcription, and/or translation. These regions of the vector genome or antigenome represent target sites for disruption or
25 modification of regulatory functions associated with introduction of the heterologous gene or genome segment.

As used herein, the term "gene" generally refers to a portion of a subject genome, e.g., a PIV genome, encoding an mRNA and typically begins at the upstream end with a gene-start (GS) signal and ends at the downstream end with the gene-end (GE) signal.
30 The term gene is also interchangeable with the term "translational open reading frame", or ORF, particularly in the case where a protein, such as the PIV C protein, is expressed from an additional ORF rather than from a unique mRNA. In the exemplary case of HPIV3, the

Alternative chimeric PIV of the invention will contain protective antigenic determinants of HPIV1, HPIV2 and/or HPIV3. This is preferably achieved by expression of one or more HN and/or F genes or genome segments by the vector PIV, or as extra or substitute genes from the heterologous donor pathogen. In certain embodiments, a HPIV3-1 or HPIV3-2 chimeric virus may be constructed for use as a vaccine or vector strain, in which the HPIV1 or HPIV2 HN and/or F genes replace their PIV3 counterpart(s) (Skiadopoulos et al., Vaccine 18:503-510, 1999; Tao et al., Vaccine 17:1100-1108, 1999; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998 (and corresponding International Application published as WO 98/53078); U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; each incorporated herein by reference). In this context, a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45L* (Skiadopoulos et al., J. Virol. 72:1762-8, 1998; Tao et al., J. Virol. 72:2955-2961, 1998; Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.*cp45L* is attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1.*cp45*, has also been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations in HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al., Vaccine 18:503-510, 1999, incorporated herein by reference).

In preferred embodiments of the invention, the chimeric PIV bear one or more major antigenic determinants of a human PIV, or against multiple human PIVs, including HPIV1, HPIV2 or HPIV3. These preferred vaccine candidates elicit an effective immune response in humans against one or more selected HPIVs. As noted above, the antigenic determinant(s) that elicit(s) an immune response against HPIV may be encoded by the vector genome or antigenome, or may be inserted within or joined to the PIV vector genome or antigenome as a heterologous gene or gene segment. The major protective antigens of human PIVs are their HN and F glycoproteins. However, all PIV genes are candidates for encoding antigenic determinants of interest, including internal protein genes which may encode such determinants as, for example, CTL epitopes.

Preferred chimeric PIV vaccine viruses of the invention bear one or more major antigenic determinants from each of a plurality of HPIVs or from a HPIV and a non-

PIV pathogen. Chimeric PIV thus constructed include a partial or complete HPIV genome or antigenome, for example of HPIV3, and one or more heterologous gene(s) or genome segment(s) encoding antigenic determinant(s) of a heterologous PIV, for example HPIV1 or HPIV2. In alternative embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV1 or HPIV2 may be added to or substituted within a partial or complete HPIV3 genome or antigenome. In various exemplary embodiments described below, both HPIV1 genes encoding the HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes in a chimeric PIV vaccine candidate. These and other constructs yield chimeric PIVs that elicit either a mono- or poly-specific immune response in humans to one or more HPIVs. Further detailed aspects of the invention are provided in United States Patent Application entitled CONSTRUCTION AND USE OF RECOMBINANT PARAINFLUENZA VIRUSES EXPRESSING A CHIMERIC GLYCOPROTEIN, filed on December 10, 1999 by Tao et al. and identified by Attorney Docket No. 17634-000340, and U.S. Patent Application entitled USE OF RECOMBINANT PARAINFLUENZA VIRUS (PIV) AS A VECTOR TO PROTECT AGAINST DISEASE CAUSED BY PIV AND RESPIRATORY SYNCYTIAL VIRUS (RSV), filed on December 10, 1999 by Murphy et al. and identified by Attorney Docket No. 17634-000330, each incorporated herein by reference.

In exemplary aspects of the invention, heterologous genes or genome segments encoding antigenic determinants from both HPIV1 and HPIV2 are added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. For instance, one or more HPIV1 genes or genome segments encoding HN and/or F glycoproteins, or antigenic determinant(s) thereof, and one or more HPIV2 genes or genome segments encoding HN and/or F glycoproteins or antigenic determinants can be added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. In one example described below, both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome. This vector construct can be further modified by addition or incorporation of one or more genes or gene segments encoding antigenic determinant(s) of HPIV2. Thus, specific constructs exemplifying the invention are provided which yield chimeric PIVs having antigenic determinants of both HPIV1 and HPIV2, as exemplified by the vaccine candidates rPIV3-1.2HN and rPIV3-1cp45.2HN described herein below.

secondary structure composed primarily of hydrophobic β sheets interconnected by random hydrophilic coil structures. The HN epitopes were located in predicted coil regions. Epitopes recognized by MAbs which inhibit neuraminidase activity of the virus were located in a region which appears to be structurally conserved among several paramyxovirus HN proteins and which may represent the sialic acid-binding site of the HN molecule.

This exemplary work, employing conventional antigenic mapping methods, identified single amino acids which are important for the integrity of HN epitopes. Most of these epitopes are located in the C-terminal half of the molecule, as expected for a protein anchored at its N terminus (Elango et al., *J. Virol.* 57:481-489, 1986). Previously published operational and topographic maps of the PIV3 HN indicated that the MAbs employed recognized six distinct groups of epitopes (I to VI) organized into two topographically separate sites (A and B), which are partially bridged by a third site (C). These groups of epitopes represent useful candidates for antigenic determinants that may be incorporated, alone or in various combinations, within chimeric PIVs of the invention. (See, also, Coelingh et al., *Virology* 143:569-582, 1985; Coelingh et al., *Virology* 162:137-143, 1988; Ray et al., *Virology* 148:232-236, 1986; Rydbeck et al., *J. Gen. Virol.* 67:1531-1542, 1986, each incorporated herein by reference),

Additional studies by van Wyke Coelingh et al. (*J. Virol.* 63:375-382, 1989) provide further information relating to selection of PIV antigenic determinants for use within the invention. In this study, twenty-six monoclonal antibodies (MAbs) (14 neutralizing and 12 nonneutralizing) were used to examine the antigenic structure, biological properties, and natural variation of the fusion (F) glycoprotein of HPIV3. Analysis of laboratory-selected antigenic variants and of PIV3 clinical isolates indicated that the panel of MAbs recognizes at least 20 epitopes, 14 of which participate in neutralization. Competitive binding assays confirmed that the 14 neutralization epitopes are organized into three nonoverlapping principal antigenic regions (A, B, and C) and one bridge site (AB), and that the 6 nonneutralization epitopes form four sites (D, E, F, and G). Most of the neutralizing MAbs were involved in nonreciprocal competitive binding reactions, suggesting that they induce conformational changes in other neutralization epitopes.

Other antigenic determinants for use within the invention have been identified and characterized for respiratory syncytial virus (RSV). For example, Beeler et al., *J. Virol.* 63:2941-2950, 1989, incorporated herein by reference, employed eighteen neutralizing

monoclonal antibodies (MAbs) specific for the fusion glycoprotein of the A2 strain of RSV to construct a detailed topological and operational map of epitopes involved in RSV neutralization and fusion. Competitive binding assays identified three nonoverlapping antigenic regions (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was then examined by using 23 clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates. These antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, all represent useful candidates for integration within chimeric PIV of the invention to elicit novel immune responses as described above. (See also, Anderson et al., J. Infect. Dis. 151:626-633, 1985; Coelingh et al., J. Virol. 63:375-382, 1989; Fenner et al., Scand. J. Immunol. 24:335-340, 1986; Fernie et al., Proc. Soc. Exp. Biol. Med. 171:266-271, 1982; Sato et al., J. Gen. Virol. 66:1397-1409, 1985; Walsh et al., J. Gen. Virol. 67:505-513, 1986, and Olmsted et al., J. Virol. 63:411-420, 1989, each incorporated herein by reference).

To express antigenic determinants of heterologous PIVs and non-PIV pathogens, the invention provides numerous human and non-human PIV vectors, including bovine PIV (BPIV) vectors. These vectors are readily modified according the recombinant methods described herein to carry heterologous antigenic determinants and elicit one or more specific humoral or cell mediated immune responses against the heterologous pathogen and vector PIV. In exemplary embodiments, one or more heterologous genes or genome segments from a donor pathogen is combined with a HPIV3 vector genome or antigenome. In other exemplary embodiments, the heterologous gene or genome segment is incorporated within a chimeric HPIV vector genome or antigenome, for example a chimeric HPIV3-1 vector genome or antigenome having one or both HPIV1 genes encoding the HN and F

glycoproteins substituted for their counterpart HPIV3 HN and/or F gene(s). In more detailed embodiments, a transcription unit comprising an open reading frame (ORF) of the measles virus HA gene is added to a HPIV3 vector genome or antigenome at various positions, yielding exemplary chimeric PIV/measles vaccine candidates rPIV3(HA HN-L), rPIV3(HA N-P), *rcp45L*(HA N-P), rPIV3(HA P-M), or *rcp45L*(HA P-M). Alternatively, chimeric PIV for vaccine use may incorporate one or more antigenic determinants of HPIV2, for example an HPIV2 HN gene, within a chimeric HPIV3-1 vector genome or antigenome.

In other detailed embodiments of the invention, chimeric PIVs are engineered that incorporate heterologous nucleotide sequences encoding protective antigens from respiratory syncytial virus (RSV) to produce infectious, attenuated vaccine candidates. The cloning of RSV cDNA and other disclosure is provided in U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application No. 09/291,894, filed on April 13, 1999; International Application No. PCT/US00/09696, filed April 12, 2000, corresponding to U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999; Collins et al., Proc Nat. Acad. Sci. U.S.A. 92:11563-11567, 1995; Bukreyev et al., J. Virol. 70:6634-41, 1996; Juhasz et al., J. Virol. 71:5814-5819, 1997; Durbin et al., Virology 235:323-332, 1997; He et al. Virology 237:249-260, 1997; Baron et al. J. Virol. 71:1265-1271, 1997; Whitehead et al., Virology 247:232-9, 1998a; Whitehead et al., J. Virol. 72:4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; and Whitehead et al., J. Virol. 73:3438-3442, 1999, and Bukreyev et al., Proc. Nat. Acad. Sci. U.S.A. 96:2367-72, 1999, each incorporated herein by reference in its entirety for all purposes). Other reports and discussion incorporated or set forth herein identify and characterize RSV antigenic determinants that are useful within the invention.

PIV chimeras incorporating one or more RSV antigenic determinants, preferably comprise a human PIV (e.g., HPIV1, HPIV2, HPIV3) vector genome or antigenome with a heterologous gene or genome segment encoding an antigenic RSV glycoprotein, protein domain (e.g., a glycoprotein ectodomain) or one or more immunogenic epitopes. In one embodiment, one or more genes or genome segments from RSV F and/or G

genes is/are combined with the vector genome or antigenome to form the chimeric PIV vaccine candidate. Certain of these constructs will express chimeric proteins, for example fusion proteins having a cytoplasmic tail and/or transmembrane domain of PIV fused to an ectodomain of RSV to yield a novel attenuated virus that elicits a multivalent immune response against both PIV and RSV

As noted above, it is often desirable to adjust the phenotype of chimeric PIV for vaccine use by introducing additional mutations that increase or decrease attenuation or otherwise alter the phenotype of the chimeric virus. Detailed descriptions of the materials and methods for producing recombinant PIV from cDNA, and for making and testing various mutations and nucleotide modifications set forth herein as supplemental aspects of the present invention are provided in, e.g., Durbin et al., *Virology* 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference. In particular, these documents describe methods and procedures for mutagenizing, isolating and characterizing PIV to obtain attenuated mutant strains (e.g., temperature sensitive (*ts*), cold passaged (*cp*) cold-adapted (*ca*), small plaque (*sp*) and host-range restricted (*hr*) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype. In conjunction with these methods, the foregoing documents detail procedures for determining replication, immunogenicity, genetic stability and protective efficacy of biologically derived and recombinantly produced attenuated human PIV in accepted model systems, including murine and non-human primate model systems. In addition, these documents describe general methods for developing and testing immunogenic compositions, including monovalent and bivalent vaccines, for prophylaxis and treatment of PIV infection. Methods for producing infectious recombinant PIV by construction and expression of cDNA encoding a PIV genome or antigenome coexpressed with essential PIV proteins are also described in the above-incorporated documents, which include description of the following exemplary plasmids that may be employed to produce infectious PIV clones: p3/7(131) (ATCC 97990); p3/7(131)2G (ATCC 97889); and p218(131) (ATCC 97991); each deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and granted the above identified accession numbers.

Also disclosed in the above-incorporated references are methods for constructing and evaluating infectious recombinant PIV that are modified to incorporate phenotype-specific mutations identified in biologically-derived PIV mutants, e.g., cold passaged (*cp*), cold adapted (*ca*), host range restricted (*hr*), small plaque (*sp*), and/or temperature sensitive (*ts*) mutants, for example the JS HPIV3 *cp* 45 mutant strain. Mutations identified in these mutants can be readily incorporated into chimeric PIV of the instant invention. In exemplary embodiments, one or more attenuating mutations occur in the polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS *cp*45. Preferably, these mutations are incorporated in chimeric PIV of the invention by an identical, or conservative, amino acid substitution as identified in the biological mutant. In more detailed aspects, chimeric PIV for vaccine use incorporate one or more mutation wherein Tyr₉₄₂ is replaced by His, Leu₉₉₂ is replaced by Phe, and/or Thr₁₅₅₈ is replaced by Ile. Substitutions that are conservative to these replacement amino acids are also useful to achieve desired attenuation in chimeric vaccine candidates. The HPIV3 JS *cp*45 strain has been deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A. under Patent Deposit Designation PTA-2419.

Other exemplary mutations that can be adopted in chimeric PIVs from biologically derived PIV mutants include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS *cp*45. In more detailed aspects, these mutations are represented as Val₉₆ to Ala or Ser₃₈₉ to Ala or substitutions that are conservative thereto. Also useful within chimeric PIV of the invention are amino acid substitution in the C protein, e.g., a mutation at a position corresponding to Ile₉₆ of JS *cp*45, preferably represented by an identical or conservative substitution of Ile₉₆ to Thr. Further exemplary mutations that can be adopted from biologically derived PIV mutants include one or more mutations in the F protein, including mutations adopted from JS *cp*45 at a position corresponding to residues Ile₄₂₀ or Ala₄₅₀ of JS *cp*45, preferably represented by acid substitutions Ile₄₂₀ to Val or Ala₄₅₀ to Thr or substitutions conservative thereto. Alternatively or in addition, chimeric PIV of the invention can adopt one or more amino acid substitutions in the HN protein, as exemplified by a mutation at a position corresponding to residue Val₃₈₄ of JS *cp*45, preferably represented by the substitution Val₃₈₄ to Ala.

Yet additional embodiments of the invention include chimeric PIV which incorporate one or more mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence, that specify desired phenotypic changes such as attenuation. Exemplary mutations in this context may be engineered at a position in the 3' leader of the chimeric virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS *cp45*. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS *cp45*. In more detailed aspects, chimeric PIV incorporate a T to C change at nucleotide 23, a C to T change at nucleotide 24, a G to T change at nucleotide 28, and/or a T to A change at nucleotide 45. Additional mutations in extragenic sequences are exemplified by an A to T change in the N gene start sequence at a position corresponding to nucleotide 62 of JS.

These foregoing exemplary mutations which can be engineered in a chimeric PIV of the invention have been successfully engineered and recovered in recombinant PIV—as represented by the recombinant PIV clones designated *rcp45*, *rcp45 L*, *rcp45 F*, *rcp45 M*, *rcp45 HN*, *rcp45 C*, *rcp45 F*, *rcp45 3'N*, *rcp3'NL*, and *rcp45 3'NCMFHN* (Durbin et al., *Virology* 235:323-332, 1997; Skiadopoulos et al., *J. Virol.* 72:1762-1768, 1998; Skiadopoulos et al., *J. Virol.* 73:1374-1381, 1999; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, the above-incorporated references describe construction of chimeric PIV recombinants, e.g., having the HN and F genes of HPIV1 substituted into a partial HPIV3 background genome or antigenome, which is further modified to bear one or more of the attenuating mutations identified in HPIV3 JS *cp45*. One such chimeric recombinant incorporates all of the attenuating mutations identified in the L gene of *cp45*. It has since been shown that all of the *cp45* mutations outside of the heterologous (HPIV1) HN and F genes can be incorporated in a HPIV3-1 recombinant to yield an attenuated, chimeric vaccine candidate.

From JS *cp45* and other biologically derived PIV mutants, a large "menu" of attenuating mutations is provided, each of which can be combined with any other mutation(s) for adjusting the level of attenuation, immunogenicity and genetic stability in

chimeric PIV of the invention. In this context, many chimeric PIVs will include one or more, and preferably two or more, mutations from biologically derived PIV mutants, e.g., any one or combination of mutations identified in JS *cp45*. Preferred chimeric PIVs within the invention will incorporate a plurality and up to a full complement of the mutations present in JS *cp45* or other biologically derived mutant PIV strains. Preferably, these mutations are stabilized against reversion in chimeric PIV by multiple nucleotide substitutions in a codon specifying each mutation.

Yet additional mutations that may be incorporated in chimeric PIV of the invention are mutations, e.g., attenuating mutations, identified in heterologous PIV or other nonsegmented negative stranded RNA viruses. In particular, attenuating and other desired mutations identified in one negative stranded RNA virus may be "transferred", e.g., copied, to a corresponding position within the genome or antigenome of a chimeric PIV. Briefly, desired mutations in one heterologous negative stranded RNA virus are transferred to the chimeric PIV recipient (either in the vector genome or antigenome or in the heterologous donor gene or genome segment). This involves mapping the mutation in the heterologous mutant virus, identifying by routine sequence alignment the corresponding site in the recipient PIV, and mutating the native sequence in the PIV recipient to the mutant genotype (either by an identical or conservative mutation), as described in International Application No. PCT/US00/09695, filed April 12, 2000, corresponding to U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999, incorporated herein by reference. As this disclosure teaches, it is preferable to modify the recipient chimeric PIV genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a similar substitution can be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will specify an identical or conservative amino acid to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the function of the wild-type residue). Negative stranded RNA viruses from which exemplary mutations are identified and transferred into a recombinant PIV of the invention include other PIVs (e.g., HPIV1, HPIV2, HPIV3, BPIV and MPIV), RSV, Sendai virus (SeV), Newcastle disease virus (NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV), rabies

virus (RaV) and vesicular stomatitis virus (VSV), among others. A variety of exemplary mutations are disclosed, including but not limited to an amino acid substitution of phenylalanine at position 521 of the RSV L protein corresponding to and therefore transferable to a substitution of phenylalanine (or a conservatively related amino acid) at position 456 of the HPIV3 L protein. In the case of mutations marked by deletions or insertions, these can be introduced as corresponding deletions or insertions into the recombinant virus, however the particular size and amino acid sequence of the deleted or inserted protein fragment can vary.

Attenuating mutations in biologically derived PIV and other nonsegmented negative stranded RNA viruses for incorporation within chimeric PIV of the invention may occur naturally or may be introduced into wild-type PIV strains by well known mutagenesis procedures. For example, incompletely attenuated parental PIV strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (*sp*) in cell culture, as described in the above incorporated references. By "biologically derived PIV" is meant any PIV not produced by recombinant means. Thus, biologically derived PIV include all naturally occurring PIV, including, e.g., naturally occurring PIV having a wild-type genomic sequence and PIV having allelic or mutant genomic variations from a reference wild-type PIV sequence, e.g., PIV having a mutation specifying an attenuated phenotype. Likewise, biologically derived PIV include PIV mutants derived from a parental PIV by, *inter alia*, artificial mutagenesis and selection procedures.

As noted above, production of a sufficiently attenuated biologically derived PIV mutant can be accomplished by several known methods. One such procedure involves subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a cp mutant or other partially attenuated PIV strain is adapted to efficient growth at a lower temperature by passage in culture. This selection of mutant PIV during cold-passage substantially reduces any residual virulence in the derivative strains as compared to the partially attenuated parent. Alternatively, specific mutations can be introduced into biologically derived PIV by subjecting a partially attenuated parent virus to chemical mutagenesis, e.g., to introduce ts mutations or, in the

case of viruses which are already ts, additional ts mutations sufficient to confer increased attenuation and/or stability of the ts phenotype of the attenuated derivative. Means for the introduction of ts mutations into PIV include replication of the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical
5 mutagens can also be used. Attenuation can result from a ts mutation in almost any PIV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene. The level of temperature sensitivity of replication in exemplary attenuated PIV for use within the invention is determined by comparing its replication at a permissive temperature with that at several restrictive temperatures. The lowest temperature
10 at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the shutoff temperature. In experimental animals and humans, both the replication and virulence of PIV correlate with the mutant's shutoff temperature.

The JS *cp45* HPIV3 mutant has been found to be relatively stable genetically,
15 highly immunogenic, and satisfactorily attenuated. Nucleotide sequence analysis of this biologically derived virus, and of recombinant viruses incorporating various individual and combined mutations found therein, indicates that each level of increased attenuation is associated with specific nucleotide and amino acid substitutions. The above-incorporated references also disclose how to routinely distinguish between silent incidental mutations and
20 those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious PIV clones. This process coupled with evaluation of phenotype characteristics of parental and derivative viruses identifies mutations responsible for such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc.

Mutations thus identified are compiled into a "menu" and are then introduced
25 as desired, singly or in combination, to adjust chimeric PIV of the invention to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc., as desired. In accordance with the foregoing description, the ability to produce infectious PIV from cDNA permits introduction of specific engineered
30 changes within chimeric PIV. In particular, infectious, recombinant PIVs are employed for identification of specific mutation(s) in biologically derived, attenuated PIV strains, for example mutations which specify *ts*, *ca*, *att* and other phenotypes. Desired mutations are thus identified and introduced into chimeric PIV vaccine strains. The capability of

producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, whereafter the phenotypes of rescued recombinant viruses containing the introduced mutations to be readily determined.

5 By identifying and incorporating specific mutations associated with desired phenotypes, e.g., a *cp* or *ts* phenotype, into infectious chimeric PIV clones, the invention provides for other, site-specific modifications at, or within close proximity to, the identified mutation. Whereas most attenuating mutations produced in biologically derived PIVs are single nucleotide changes, other "site specific" mutations can also be incorporated by
10 recombinant techniques into a chimeric PIV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from 1 to 3, up to about 5-15 or more altered nucleotides (e.g., altered from a wild-type PIV sequence, from a sequence of a selected mutant PIV strain, or from a parent recombinant PIV clone subjected to mutagenesis). Such site-specific mutations may be incorporated at, or within the region of, a
15 selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within a PIV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc. Site-specific PIV mutants typically retain a desired attenuating phenotype, but may additionally exhibit altered phenotypic characteristics unrelated to attenuation, e.g.,
20 enhanced or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant PIV designed to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant PIV clone, yielding a PIV
25 with greater genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream (N-terminal direction) or downstream (C-terminal direction), e. g., from 1 to 3, 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, e.g., to construct or ablate an existing cis-acting regulatory element.

30 In addition to single and multiple point mutations and site-specific mutations, changes to the chimeric PIV disclosed herein include deletions, insertions, substitutions or rearrangements of one or more gene(s) or genome segment(s). Particularly useful are deletions involving one or more gene(s) or genome segment(s), which deletions have been

shown to yield additional desired phenotypic effects. Thus, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference, describes methods and compositions whereby expression of one or more HPIV genes, for example one or more of the C, D, and/or V ORFs, is reduced or ablated by modifying the PIV genome or antigenome to incorporate a mutation that alters the coding assignment of an initiation codon or mutation(s) that introduce one or one or more stop codon(s). Alternatively, one or more of the C, D, and/or V ORFs can be deleted in whole or in part to render the corresponding protein(s) partially or entirely non-functional or to disrupt protein expression altogether. Chimeric PIV having such mutations in C, D, and/or V, or other non-essential gene(s), possess highly desirable phenotypic characteristics for vaccine development. For example, these modifications may specify one or more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, and (v) a change in immunogenicity. One exemplary "knock out" mutant PIV lacking C ORF expression, designated rC-KO, was able to induce a protective immune response against wild type HPIV3 challenge in a non-human primate model despite its beneficial attenuation phenotype.

Thus, in more detailed aspects of the instant invention, chimeric PIV incorporate deletion or knock out mutations in a C, D, and/or V ORF(s) or other non-essential gene which alters or ablates expression of the selected gene(s) or genome segment(s). This can be achieved, e.g., by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE transcription signals to alter phenotype, or modifying an RNA editing site (e.g., growth, temperature restrictions on transcription, etc.). In more detailed aspects of the invention, chimeric PIVs are provided in which expression of one or more gene(s), e.g., a C, D, and/or V ORF(s), is ablated at the translational or transcriptional level without deletion of the gene or of a segment thereof, by, e.g., introducing multiple translational termination codons into a translational open reading frame (ORF), altering an initiation codon, or modifying an editing site. These forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, these methods provide yet additional, novel types of attenuating mutations which ablate expression of a viral gene that is not one of the major viral protective antigens. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be

alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Several other gene knock-outs for the C, D, and/or V ORF(s) deletion and knock out mutants can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer et al., Virology 216:309-316, 1996; Radecke et al., Virology 217:418-421, 1996; Kato et al., EMBO J. 16:578-587, 1987; and Schneider et al., Virology 277:314-322, 1996, each incorporated herein by reference).

Nucleotide modifications that may be introduced into chimeric PIV constructs of the invention may alter small numbers of bases (e.g., from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (e.g., 50-100, 100-300, 300-500, 500-1,000 bases), or nearly complete or complete genes (e.g., 1,000-1,500 nucleotides, 1,500-2,500 nucleotides, 2,500-5,000, nucleotides, 5,00-6,5000 nucleotides or more) in the vector genome or antigenome or heterologous, donor gene or genome segment, depending upon the nature of the change (i.e., a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large block(s) of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In related aspects, the invention provides for supplementation of mutations adopted into a chimeric PIV clone from biologically derived PIV, e.g., *cp* and *ts* mutations, with additional types of mutations involving the same or different genes in a further modified PIV clone. Each of the PIV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a chimeric PIV exhibiting novel vaccine characteristics. Thus, in addition to or in combination with attenuating mutations adopted from biologically derived PIV mutants, the present invention also provides a range of additional methods for attenuating or otherwise modifying the phenotype of a chimeric PIV based on recombinant engineering of infectious PIV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a chimeric PIV genome or antigenome for incorporation into infectious clones. More specifically, to achieve desired structural and phenotypic changes in recombinant PIV, the invention allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or nucleotide sequence from a parent genome or antigenome, as well as mutations which delete,

substitute, introduce or rearrange whole gene(s) or genome segment(s), within a chimeric PIV clone.

Thus provided are modifications in chimeric PIV of the invention which simply alter or ablate expression of a selected gene, e.g., by introducing a termination codon within a selected PIV coding sequence or altering its translational start site or RNA editing site, changing the position of a PIV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (e.g., by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (e.g., growth, temperature restrictions on transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected gene(s), or translation of selected protein(s). In this context, any PIV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant PIV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. As for coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, e.g., by the use of minireplicons and recombinant PIV.

In addition, a variety of other genetic alterations can be produced in a PIV genome or antigenome for incorporation into a chimeric PIV, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV, e.g., to adjust growth, attenuation, immunogenicity, genetic stability or provide other advantageous structural and/or phenotypic effects. These additional types of mutations are also disclosed in the foregoing incorporated references and can be readily engineered into chimeric PIV of the invention. For example, restriction site markers are routinely introduced within chimeric PIVs to facilitate cDNA construction and manipulation.

In addition to these changes, the order of genes in a chimeric PIV construct can be changed, a PIV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into chimeric PIV constructs of the invention include mutations directed toward cis-acting signals, which can be readily identified, e.g., by mutational analysis of PIV minigenomes. For example, insertional and deletional analysis of the leader and trailer and flanking sequences identifies viral promoters and transcription signals and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also has identified many mutations which affect RNA replication or transcription. Any of these mutations can be inserted into a chimeric PIV antigenome or genome as described herein. Evaluation and manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of PIV minigenomes as described in the above-incorporated references.

Additional mutations within chimeric PIVs of the invention may also include replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific PIV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Haas et al., Current Biol. 6:315-324, 1996, incorporated herein by reference). Optimization by recombinant methods of the codon usage of the mRNAs encoding the HN and F proteins of PIV will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected PIV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate PIV gene expression by specifying up- or down-regulation of translation. Alternatively, or in combination with other recombinant modifications disclosed herein, gene expression of a chimeric PIV can be modulated by altering a transcriptional GS or GE signal of any selected gene(s) of the virus. In alternative embodiments, levels of gene expression in a chimeric PIV vaccine candidate are modified at the level of transcription. In one aspect, the position of a selected gene in the PIV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be

achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild-type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel chimeric PIV vector virus having
5 attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as increased antigen expression.

In other embodiments, chimeric PIVs useful in vaccine formulations can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the
10 modification will be in the HN and/or F proteins. An entire HN or F gene, or a genome segment encoding a particular immunogenic region thereof, from one PIV strain or group is incorporated into a chimeric PIV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different PIV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented. Progeny
15 virus produced from the modified PIV clone can then be used in vaccination protocols against emerging PIV strains.

Replacement of a human PIV coding sequence or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a heterologous counterpart yields chimeric PIV having a variety of possible attenuating and other
20 phenotypic effects. In particular, host range and other desired effects arise from substituting a bovine PIV (BPIV) or murine PIV (MPIV) protein, protein domain, gene or genome segment imported within a human PIV background, wherein the bovine or murine gene does not function efficiently in a human cell, e.g., from incompatibility of the heterologous sequence or protein with a biologically interactive human PIV sequence or protein (i.e., a
25 sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, bovine PIV sequences are selected for introduction into human PIV based on known aspects of bovine and human
30 PIV structure and function.

In more detailed aspects, the invention provides methods for attenuating chimeric PIV vaccine candidates based on the further construction of chimeras between

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This mode of attenuation contrasts sharply to HPIV vaccine candidates that are attenuated by one or more point mutations, where reversion of an individual mutation may yield a significant or complete reacquisition of virulence. In addition, several known attenuating point mutations in HPIV typically yield a temperature sensitive phenotype. One problem with attenuation associated with temperature sensitivity is that the virus can be overly restricted for replication in the lower respiratory tract while being under attenuated in the upper respiratory tract. This is because there is a temperature gradient within the respiratory tract, with temperature being higher (and more restrictive) in the lower respiratory tract and lower (less restrictive) in the upper respiratory tract. The ability of an attenuated virus to replicate in the upper respiratory tract can result in complications including congestion, rhinitis, fever and otitis media. Thus, attenuation achieved solely by temperature sensitive mutations may not be ideal. In contrast, host range mutations present in chimeric PIV of the invention will not in most cases confer temperature sensitivity. Therefore, the novel method of PIV attenuation provided by these kinds of modifications will be more stable genetically and phenotypically and less likely to be associated with residual virulence in the upper respiratory tract compared to other known PIV vaccine candidates.

The above-incorporated reference discloses that both Ka and SF HPIV3/BPIV3 chimeric recombinants are viable and replicate as efficiently in cell culture as either HPIV3 or BPIV3 parent—indicating that the chimeric recombinants did not exhibit gene incompatibilities that restricted replication *in vitro*. This property of efficient replication *in vitro* is important since it permits efficient manufacture of this biological. Also, the Ka and the SF HPIV3/BPIV3 chimeric recombinants (termed cKa and cSF), bearing only one bovine gene, are nearly equivalent to their BPIV3 parents in the degree of host range restriction in the respiratory tract of the rhesus monkey. In particular, the cKa and cSF viruses exhibit approximately a 60-fold or 30-fold reduction, respectively, in replication in the upper respiratory tract of rhesus monkeys compared to replication of HPIV3. Based on this finding, it is expected that other BPIV3 genes will also confer desired levels of host range restriction within chimeric PIV of the invention. Thus, according to the methods herein, a list of attenuating determinants will be readily identified in heterologous genes and genome segments of BPIV and other non-human PIVs that will confer, in appropriate combination, a desired level of host range restriction and immunogenicity on chimeric PIV selected for vaccine use.

background genome or antigenome is an HPIV genome or antigenome, e.g., an HPIV3, HPIV2, or HPIV1 background genome or antigenome, to which is added or into which is substituted one or more BPIV gene(s) or genome segment(s), preferably from BPIV3. In one exemplary embodiment described below, an ORF of the N gene of a BPIV3 is substituted for that of an HPIV. Alternatively, the background genome or antigenome may be a BPIV genome or antigenome which is combined with one or more genes or genome segments encoding a HPIV3, HPIV2, or HPIV1 glycoprotein, glycoprotein domain or other antigenic determinant.

In accordance with the methods of the invention, any BPIV gene or genome segment, singly or in combination with one or more other BPIV genes, can be combined with HPIV sequences to give rise to a human-bovine chimeric PIV vaccine candidate. Any HPIV, including different strains of a particular HPIV serotype, e.g., HPIV3 will be a reasonable acceptor for attenuating BPIV gene(s). In general, the HPIV3 gene(s) or genome segment(s) selected for inclusion in a human-bovine chimeric PIV for use as a vaccine against human PIV will include one or more of the HPIV protective antigens such as the HN or F glycoproteins.

In exemplary aspects of the invention, human-bovine chimeric PIV bearing one or more bovine gene(s) or genome segment(s) exhibits a high degree of host range restriction, e.g., in the respiratory tract of mammalian models of human PIV infection such as non-human primates. In exemplary embodiments a human PIV is attenuated by the addition or substitution of one or more bovine gene(s) or genome segment(s) to a partial or complete human, e.g., HPIV3, PIV background genome or antigenome. In one example, the HPIV3 N gene is substituted by the BPIV3 N gene to yield a novel human-bovine chimeric PIV vector, and within this vector the measles HA gene is substituted to yield a multivalent, HPIV/measles vaccine candidate, as exemplified by the recombinant rHPIV3-N_B HA_{P-M} described below.

Preferably, the degree of host range restriction exhibited by human-bovine chimeric PIV vectors for developing vaccine candidates of the invention is comparable to the degree of host range restriction exhibited by the respective BPIV parent or "donor" strain. Preferably, the restriction should have a true host range phenotype, i.e., it should be specific to the host in question and should not restrict replication and vaccine preparation *in vitro* in a suitable cell line. In addition, human-bovine chimeric PIV vectors bearing one or more

bovine gene(s) or genome segment(s) elicit a high level of resistance in hosts susceptible to PIV infection. Thus, the invention provides a new basis for attenuating a live virus vector for developing vaccines against PIV and other pathogens, based on host range effects.

In related aspects of the invention, human-bovine chimeric PIV vectors
5 comprise a BPIV recipient or backbone virus that incorporates one or more heterologous gene(s) that encode an HPIV HN and/or F glycoprotein(s). Alternatively, the chimeric PIV may incorporate one or more genome segment(s) encoding an ectodomain (and alternatively a cytoplasmic domain and/or transmembrane domain), or immunogenic epitope of an HPIV HN and/or F glycoprotein(s). These immunogenic proteins, domains and epitopes are
10 particularly useful within human-bovine chimeric PIV because they generate novel immune responses in an immunized host. In particular, the HN and F proteins, and immunogenic domains and epitopes therein, provide major protective antigens.

In certain embodiments of the invention, addition or substitution of one or more immunogenic gene(s) or genome segment(s) from a human PIV subgroup or strain to
15 or within a bovine background, or recipient, genome or antigenome yields a recombinant, chimeric virus or subviral particle capable of generating an immune response directed against the human donor virus, including one or more specific human PIV subgroups or strains, while the bovine backbone confers an attenuated phenotype making the chimera a useful candidate for vaccine development. In one exemplary embodiment, one or more
20 human PIV glycoprotein genes, e.g., HN and/or F, are added to or substituted within a partial or complete bovine genome or antigenome to yield an attenuated, infectious human-bovine chimera that elicits an anti-human PIV immune response in a susceptible host. Within one such exemplary vector (carrying the HPIV3 JS HN and F glycoprotein genes in BPIV3 background), the RSV A glycoprotein genes G and F were successfully inserted as additional
25 heterologous ORF to yield multivalent, HPIV/RSV vaccine candidates exemplified by the recombinant viruses rB/HPIV3-G1 and rB/HPIV3-F1 described below.

In alternate embodiments, human-bovine chimeric PIV vectors additionally incorporate a gene or genome segment encoding an immunogenic protein, protein domain or epitope from multiple human PIV strains, for example two HN or F proteins or
30 immunogenic portions thereof each from a different HPIV, e.g., HPIV1 or HPIV2. Alternatively, one glycoprotein or immunogenic determinant may be provided from a first HPIV, and a second glycoprotein or immunogenic determinant may be provided from a

second HPIV by substitution without the addition of an extra glycoprotein- or determinant-
encoding polynucleotide to the genome or antigenome. Substitution or addition of HPIV
glycoproteins and antigenic determinants may also be achieved by construction of a genome
or antigenome that encodes a chimeric glycoprotein in the recombinant virus or subviral
5 particle, for example having an immunogenic epitope, antigenic region or complete
ectodomain of a first HPIV fused to a cytoplasmic domain of a heterologous HPIV. For
example, a heterologous genome segment encoding a glycoprotein ectodomain from a
HPIV1 or HPIV2 HN or F glycoprotein may be joined with a genome segment encoding a
corresponding HPIV3 HN or F glycoprotein cytoplasmic/endodomain in the background
10 genome or antigenome.

In alternate embodiments a human-bovine chimeric PIV vector genome or
antigenome may encode a substitute, extra, or chimeric glycoprotein or antigenic
determinant thereof in the recombinant virus or subviral particle, to yield a viral recombinant
having both human and bovine glycoproteins, glycoprotein domains, or immunogenic
15 epitopes. For example, a heterologous genome segment encoding a glycoprotein ectodomain
from a human PIV HN or F glycoprotein may be joined with a genome segment encoding a
corresponding bovine HN or F glycoprotein cytoplasmic/endodomain in the background
genome or antigenome. Alternatively, the human PIV HN or F glycoprotein or parts thereof
may be joined with a genome segment encoding an HN or F glycoprotein or parts thereof
20 from another PIV strain or serotype.

In combination with the host range phenotypic effects provided in the human-
bovine chimeric PIV of the invention, it is often desirable to adjust the attenuation phenotype
by introducing additional mutations that increase or decrease attenuation of the chimeric
virus. Thus, in additional aspects of the invention, attenuated, human-bovine chimeric PIV
25 vectors are produced in which the chimeric genome or antigenome is further modified by
introducing one or more attenuating mutations specifying an attenuating phenotype in the
resultant virus or subviral particle. These can include mutations in RNA regulatory
sequences or in encoded proteins. These attenuating mutations may be generated *de novo*
and tested for attenuating effects according to a rational design mutagenesis strategy.
30 Alternatively, the attenuating mutations may be identified in existing biologically derived
mutant PIV and thereafter incorporated into a human-bovine chimeric PIV of the invention.

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In preferred chimeric vaccine candidates of the invention, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model for PIV replication in humans, e.g., hamsters or rhesus monkeys, may be reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-100-fold and up to 1,000-fold or greater overall (e.g., as measured between 3-8 days following infection) compared to growth of the corresponding wild-type or mutant parental PIV strain.

Infectious chimeric PIV vector clones of the invention can also be engineered according to the methods and compositions disclosed herein to enhance immunogenicity and induce a level of protection greater than that provided by infection with a wild-type, parental (i.e., vector or heterologous donor) PIV or non-PIV pathogen . For example, one or more supplemental immunogenic epitope(s), protein domains, or proteins from a heterologous PIV strain or type, or from a non-PIV pathogen such as measles or RSV, can be added to a chimeric PIV by appropriate nucleotide changes in the chimeric genome or antigenome. Alternatively, chimeric PIVs of the invention can be engineered to add or ablate (e.g., by amino acid insertion, substitution or deletion) immunogenic proteins, protein domains, or forms of specific proteins associated with desirable or undesirable immunological reactions.

Within the methods of the invention, additional genes or genome segments may be inserted into or proximate to the chimeric PIV vector genome or antigenome. These genes may be under common control with recipient genes, or may be under the control of an independent set of transcription signals. In addition to genes and genome segments encoding antigenic determinants, genes of interest in this context include genes encoding cytokines, for example, an interleukin (e.g., interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL6), interleukin 18 (IL-18)), tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), or granulocyte-macrophage colony stimulating factor (GM-CSF), as well as IL-2 through IL-18, especially IL-2, IL-6 and IL-12, and IL-18, gamma-interferon (see, e.g., U.S. Application No. 09/614,285, filed July 12, 2000, corresponding to U.S. Provisional Application Serial No. 60/143,425 filed July 13, 1999, incorporated herein by reference). Coexpression of these additional proteins provides the ability to modify and improve immune responses against chimeric PIV of the invention both quantitatively and qualitatively.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within chimeric PIV of the invention yield highly

stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (i.e., not essential for *in vitro* growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., EMBO. J. 16:578-87, 1997, incorporated herein by reference). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

Introduction of the foregoing defined mutations into an infectious, chimeric PIV clone can be achieved by a variety of well known methods. By "infectious clone" with regard to DNA is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of serving as template to produce the genome of an infectious virus or subviral particle. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA subfragments to assemble a complete antigenome or genome cDNA as described herein has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or any subfragment thereof, can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, CA) or a method using a double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and available for use in producing the mutations of interest in the PIV antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Thus, in one illustrative embodiment mutations are introduced by using the Muta-gene phagemid *in vitro* mutagenesis kit available from Bio-Rad. In brief, cDNA encoding a portion of a PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies, Gaithersburg, MD). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are

designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome fragment is then amplified and the mutated piece is then reintroduced into the full-length genome or antigenome clone.

5 The invention also provides methods for producing infectious chimeric PIV from one or more isolated polynucleotides, e.g., one or more cDNAs. According to the present invention cDNA encoding a PIV genome or antigenome is constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which
10 serves as the template for the synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome, corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of the complementing sequences that encode proteins necessary to generate a transcribing, replicating nucleocapsid, i.e., sequences that
15 encode N, P, and L proteins.

For purposes of the present invention the genome or antigenome of the recombinant PIV of the invention need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a
20 gene may be provided by complementation or the like from a separate nucleotide molecule, or can be expressed directly from the genome or antigenome cDNA.

By recombinant PIV is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced therefrom. The recombinant expression system will
25 employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

30 To produce infectious PIV from cDNA-expressed genome or antigenome, the genome or antigenome is coexpressed with those PIV proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent

for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Infectious PIV of the invention are produced by intracellular or cell-free
5 coexpression of one or more isolated polynucleotide molecules that encode a PIV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins necessary to generate a transcribing, replicating nucleocapsid. Among the viral proteins useful for coexpression to yield infectious PIV are the major nucleocapsid protein (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein, fusion protein (F),
10 hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M) protein. Also useful in this context are products of the C, D and V ORFs of PIV.

cDNAs encoding a PIV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which
15 serves as a template for synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid.

20 In some embodiments of the invention the genome or antigenome of a recombinant PIV (rPIV) need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule. In
25 other embodiments, the PIV genome or antigenome encodes all functions necessary for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

By "recombinant PIV" is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from
30 virus or subviral particles produced therefrom. The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory

role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV genome or antigenome, the genome or antigenome is coexpressed with those PIV N, P and L proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Synthesis of PIV antigenome or genome together with the above-mentioned viral proteins can also be achieved *in vitro* (cell-free), e.g., using a combined transcription-translation reaction, followed by transfection into cells. Alternatively, antigenome or genome RNA can be synthesized *in vitro* and transfected into cells expressing PIV proteins.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating PIV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded by the PIV cDNA. For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the PIV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the PIV cDNA to provide antigenic diversity from the helper virus, such as in the HN or F glycoprotein genes.

In alternate embodiments of the invention, the N, P, L and other desired PIV proteins are encoded by one or more non-viral expression vectors, which can be the same or separate from that which encodes the genome or antigenome. Additional proteins may be included as desired, each encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a promoter for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an

expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., Virology 210: 202-205, 1995, incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of
5 preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Protocols: A Guide to Methods and Applications,
10 Innis et al., eds., Academic Press, San Diego, 1990, each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or
15 DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators.
20 The ribozyme can be hammerhead type (e.g., Grosfeld et al., J. Virol. 69:5677-5686, 1995), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., Nature 350:434-436, 1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-PIV nucleotides. The left- and right-hand ends are then joined via a common restriction
25 site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as
30 site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome include reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. U.S.A. 91:5695-5699, 1994), incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two
5 pieces. In other embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical
10 insertion, transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456,
15 1973), electroporation (Neumann et al., EMBO J. 1:841-845, 1982), DEAE-dextran mediated transfection (Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987), cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15:73-79, 1993) or a commercially available transfection reagent, e.g., LipofectACE® (Life Technologies) or the like (each of the foregoing references are incorporated herein by
20 reference in its entirety).

As noted above, in some embodiments of the invention the N, P, L and other desired PIV proteins are encoded by one or more helper viruses which is phenotypically distinguishable from that which encodes the genome or antigenome. The N, P, L and other desired PIV proteins can also be encoded by one or more expression vectors which can be
25 the same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as desired, encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome.

By providing infectious clones of PIV the invention permits a wide range of
30 alterations to be recombinantly produced within the PIV genome (or antigenome), yielding defined mutations which specify desired phenotypic changes. By "infectious clone" is meant cDNA or its product, synthetic or otherwise, RNA capable of being directly incorporated

into infectious virions which can be transcribed into genomic or antigenomic RNA capable of serving as a template to produce the genome of infectious viral or subviral particles. As noted above, defined mutations can be introduced by a variety of conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA subjects provide for better ease of manipulation than large cDNA subjects, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or a template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a PIV antigenome or genome cDNA of the invention.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid *in vitro* mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding an PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, e.g., cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, e.g., sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of

genome segments encoding small functional domains of proteins, e.g., epitopic sites, to about 50, 75, 100, 200-500, and 500-1,500 or more nucleotides.

The ability to introduce defined mutations into infectious PIV has many applications, including the manipulation of PIV pathogenic and immunogenic mechanisms.

5 For example, the functions of PIV proteins, including the N, P, M, F, HN, and L proteins and C, D and V ORF products, can be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the invention. The effects
10 of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing PIV minigenomes (Dimock et al., *J. Virol.* 67: 2772-8, 1993, incorporated herein by reference in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.

15 Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant PIV of the invention (e.g., substitutions of a genome segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or functional
20 relation to an existing, "counterpart" gene or genome segment from the same or different PIV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild-type or parental PIV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV. Other
25 exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, genome segments, proteins or protein regions, are typically from heterologous sources (e.g., from different PIV genes, or representing the same (i.e., homologous or allelic) gene or genome segment in different PIV
30 types or strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic

site or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant PIV within the invention, often share substantial sequence identity with a selected polynucleotide "reference sequence," e.g., with another selected counterpart sequence. As used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, (Adv. Appl. Math. 2:482, 1981), by the homology alignment algorithm of Needleman & Wunsch, (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson & Lipman, (Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988) (each of which is incorporated by reference), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI, incorporated herein by reference), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned

containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein follow conventional usage (Immunology - A Synthesis, 2nd ed.,

To select candidate vaccine viruses according to the invention, the criteria of viability, attenuation and immunogenicity are determined according to well known methods. Viruses which will be most desired in vaccines of the invention must maintain viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a vaccinee sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus. The recombinant PIV of the invention are not only viable and more appropriately attenuated than previous vaccine candidates, but are more stable genetically *in vivo*--retaining the ability to stimulate a protective immune response and in some instances to expand the protection afforded by multiple modifications, e.g., induce protection against different viral strains or subgroups, or protection by a different immunologic basis, e.g., secretory versus serum immunoglobulins, cellular immunity, and the like.

Recombinant PIV of the invention can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus (e.g., a multiply attenuated, biologically derived or recombinant PIV) is tested, e.g., for temperature sensitivity of virus replication, i.e. its phenotype, and for the small plaque or other desired phenotype. Modified viruses are further tested in animal models of PIV infection. A variety of animal models have been described and are summarized in various references incorporated herein. PIV model systems, including rodents and non-human

primates, for evaluating attenuation and immunogenic activity of PIV vaccine candidates are widely accepted in the art, and the data obtained therefrom correlate well with PIV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the invention also provides isolated, infectious recombinant PIV compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to PIV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium where it can be propagated and characterized in a controlled setting. For example, attenuated PIV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For vaccine use, recombinant PIV produced according to the present invention can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg⁺⁺ and HEPES, with or without adjuvant, as further described below.

PIV vaccines of the invention contain as an active ingredient an immunogenically effective amount of PIV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPL™ (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12

(Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV proteins, e.g., F and HN glycoproteins. As a result of the vaccination with an immunogenically effective amount of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinating strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^6 PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be combined with viruses of other PIV serotypes or strains to achieve protection against multiple PIV serotypes or strains. Alternatively, protection against multiple PIV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they may also be administered separately. Immunization with one strain may protect against different strains of the same or different serotype.

In some instances it may be desirable to combine the PIV vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. In another aspect of the invention the PIV can be employed as a vector for protective antigens of other pathogens, such as respiratory syncytial virus (RSV) or measles virus, by incorporating the sequences encoding those protective antigens into the PIV genome or antigenome which is used to produce infectious PIV, as described herein.

In all subjects, the precise amount of recombinant PIV vaccine administered, and the timing and repetition of administration, will be determined based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc.

Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6 PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated PIV sufficient to effectively stimulate or induce an anti-PIV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at levels approximately 10-fold or more lower than wild-type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated PIV.

In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) PIV infection. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be indicated for administration to different recipient groups. For example, an engineered PIV strain expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

PIV vaccines produced in accordance with the present invention can be combined with viruses expressing antigens of another subgroup or strain of PIV to achieve protection against multiple PIV subgroups or strains. Alternatively, the vaccine virus may incorporate protective epitopes of multiple PIV strains or subgroups engineered into one PIV clone, as described herein.

The PIV vaccines of the invention elicit production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild-type PIV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup.

Preferred PIV vaccine candidates of the invention exhibit a very substantial diminution of virulence when compared to wild-type virus that is circulating naturally in humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the vaccinated or incidental host do not occur.

The level of attenuation of PIV vaccine candidates may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type PIV or other attenuated PIV which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be

sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods for determining levels of PIV in the nasopharynx of an infected host are well known in the literature.

Levels of induced immunity provided by the vaccines of the invention can also be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on these measurements, vaccine dosages can be adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be advantageous for different recipient groups. For example, an engineered PIV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In yet another aspect of the invention the PIV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant PIV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls PIV expression. The infectious PIV produced by coexpressing the recombinant PIV genome or antigenome with the N, P, L and other desired PIV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant PIV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products which may be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

The following examples are provided by way of illustration, not limitation. These examples document construction of representative chimeric PIVs bearing one or more heterologous antigenic determinant(s) according to the above described methods. In one example, the HA gene of the measles virus is inserted as an extra gene into one of three gene junctions of a JS wild type or attenuated strain of HPIV3, namely, the N/P, P/M, or HN/L junction, and recombinant chimeric viruses were recovered. Insertion of the measles HA

gene at three different positions in the HPIV3 genome illustrates the range of useful constructs for transferring antigenic determinants from foreign pathogens into PIV vectors. Further, it is expected that inserted gene units that are more 3'-leader proximal will be transcribed and expressed at higher levels than the same gene units located more distally, which will allow for closer modulation of heterologous gene expression (Collins et al., 3rd ed. In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996).

The chimeric rHPIV bearing the measles virus HA insertion in a wild type rHPIV3 background replicated efficiently *in vitro* but was restricted in replication in hamsters compared to that of the rHPIV3 virus from which it was derived. Similarly, the recombinant chimeric HPIV3 bearing the measles virus HA insertion in an attenuated rHPIV3 background replicated *in vitro* and in hamsters to a level that was also slightly less than that of the attenuated rHPIV3*cp45L* mutant virus from which it was derived. The amount of HA protein expressed by cells infected with the attenuated rHPIV3-measles virus HA recombinants with the HA gene in the N/P or P/M junction was very high and even exceeded that seen in cells infected with native measles virus. The level of replication of the rHPIV3*cp45L* with a measles virus HA insert in the N/P or P/M junction was 10-fold lower in the upper respiratory tract of the hamster than that of the rHPIV3-*cp45L* parent virus indicating that gene insertions can unexpectedly contribute to the attenuation of an HPIV3 vector. These results which identify a unique host range phenotype are unexpected.

Importantly, infection of hamsters with each recombinant chimeric virus tested induced high levels of antibody to both HPIV3 and to measles virus. Animals immunized with the attenuated recombinant chimeric HPIV3 carrying the HA insertion were highly resistant to replication of HPIV3 challenge virus. While the wild type measles virus does not replicate efficiently in hamsters and thus cannot be used in challenge study, the protective efficacy of the attenuated recombinant chimeric vaccine is readily apparent from the high levels of neutralizing antibody induced. These levels are associated with a high level of resistance to measles in humans (Chen et al., J. Infect. Dis. 162:1036-42, 1990).

It is further demonstrated in the examples that attenuated chimeric recombinant HPIV vectors, combining a backbone of HPIV3 and one or more antigenic determinants of HPIV1, can also be used as vectors to express additional foreign antigens

(e.g., of HPIV2 or a non-PIV virus). This aspect of the invention takes advantage of the efficient growth and excellent attenuation properties of the HPIV3 backbone to carry antigenic determinants of multiple heterologous pathogens, as exemplified by HPIV1 and HPIV2. The cDNA encoding rPIV3-1 (a non-attenuated recombinant bearing major antigens of HPIV1) or rPIV3-1*cp45* (an attenuated recombinant bearing HPIV1 major antigens) was modified by the insertion of a gene unit containing the ORF of HPIV2 HN gene between the gene units containing the F and HN ORFs of HPIV1. The recombinant chimeric viruses, designated rPIV3-1.2HN and rPIV3-1*cp45*.2HN, were readily recovered and replicated efficiently in tissue culture. Each virus exhibited a level of temperature sensitivity of replication *in vitro* similar to that of its rPIV3-1 or rPIV3-1*cp45* parent virus. The insertion of the PIV2 HN attenuated both the rPIV3-1 and rPIV3-*cp45* viruses in hamsters, a finding similar to that observed with the insertion of the measles viruses HA into rJS and into rPIV3*cp45*. Infection of hamsters with these antigenic rPIV3-1 recombinants bearing the PIV2 HN gene insert induced serum antibody responses reactive against both HPIV1 and HPIV2.

Thus, it is possible to use an attenuated rHPIV3 or rHPIV3-1 vaccine candidate as a vector to infect the respiratory tract of susceptible hosts and thereby induce a vigorous antibody response to foreign protective antigens expressed from an extra gene unit, as well as against the HPIV vector itself. The presence of three antigenic serotypes of HPIV, which do not provide significant cross-protection, allows for more effective, sequential immunization of human infants with antigenically distinct variants of HPIV each bearing the same or different heterologous antigenic determinant(s), e.g., a protective antigen, antigenic domain or epitope of measles virus or of one or more different viral or microbial pathogens. Sequential immunization permits development of a primary immune response to the foreign protein, which is boosted during subsequent infections with a secondary, antigenically-distinct HPIV bearing one or more heterologous antigenic determinants, e.g., a protective antigen, antigenic domain or epitope of measles virus or of one or more different viral or microbial pathogens. In this way, the immunity induced to one HPIV vector can be circumvented by boosting with an antigenically distinct HPIV vector. In this context, successful immunization of animals that are immune to PIV3 has been achieved with attenuated PIV3-1 vaccine candidates, confirming the feasibility of sequential immunization with serotypically distinct PIV viruses even if these PIVs share proteins other than HN and F. (Tao et al., *Vaccine* 17:1100-8, 1999). In this study, the immunogenicity and efficacy of rPIV3-1.*cp45L* against PIV1 challenge was examined in hamsters with and without prior

immunity to PIV3. rPIV3-1.cp45L efficiently infected hamsters previously infected with wild type or attenuated PIV3, but there was approximately a five-fold reduction in replication of rPIV3-1.cp45L virus in the PIV3-immune animals. However, rPIV3-1.cp45L immunization of PIV3-immune animals induced a vigorous serum antibody response to PIV1 and reduced replication of PIV1 challenge virus 1000-fold in the lower respiratory tract and 200-fold in the upper respiratory tract. These results demonstrate that the recombinant chimeric rPIV3-1.cp45L candidate vaccine can induce immunity to PIV1 even in animals immune to PIV3. This establishes the feasibility of employing a sequential immunization schedule in which a recombinant chimeric rPIV3-1.cp45L or other PIV vaccine virus is given following a live attenuated PIV3 vaccine. since rPIV3-1.cp45L readily induced protective immunity against itself, it would also induce an effective immune response to any vectored protective antigen that it was carrying. Also, the PIVs and RSV have the unusual property of being able to reinfect the respiratory tract, although reinfections typically are not associated with serious disease. Thus, vector based vaccine constructs of the invention are useful to boost immune responses by a second, third or fourth administration of the same HPIV vector or by sequential use of different vectors.

In preferred sequential vaccination methods of the invention, it is desirable to sequentially immunize an infant with different PIV vectors each expressing the same heterologous antigenic determinant such as the measles virus HA. This sequential immunization permits the induction of the high titer of antibody to the heterologous protein that is characteristic of the secondary antibody response. In one embodiment, early infants (e.g. 2-4 month old infants) are immunized with an attenuated chimeric HPIV3 expressing a heterologous antigenic determinant, for example the measles virus HA protein, and also adapted to elicit an immune response against HPIV3. One exemplary vaccine candidate useful in this context is the rcp45L(HA P-M) recombinant. Subsequently, e.g., at four months of age the infant is again immunized but with a different, secondary PIV vector construct antigenically distinct from the first. An exemplary vaccine candidate in this context is the rPIV3-1 cp45L virus expressing the measles virus HA gene and HPIV1 antigenic determinants as functional, obligate glycoproteins of the vector. Following the first vaccination, the vaccinee will elicit a primary antibody response to both the PIV3 HN and F proteins and to the measles virus HA protein, but not to the PIV1 HN and F protein. Upon secondary immunization with the rPIV3-1 cp45L expressing the measles virus HA, the vaccinee will be readily infected with the vaccine because of the absence of antibody to the PIV1 HN and F proteins and will develop both a primary antibody response to the PIV1 HN

and F protective antigens and a high titered secondary antibody response to the heterologous measles virus HA protein. A similar sequential immunization schedule can be developed where immunity is sequentially elicited against HPIV3 and then HPIV2 by one or more of the chimeric vaccine viruses disclosed herein, simultaneous with stimulation of an initial and then secondary, high titer protective response against measles or another non-PIV pathogen. This sequential immunization strategy, preferably employing different serotypes of PIV as primary and secondary vectors, effectively circumvents immunity that is induced to the primary vector, a factor ultimately limiting the usefulness of vectors with only one serotype.

Further in accordance with this aspect of the invention, exemplary coordinate vaccination protocols may incorporate two, three, four and up to six or more separate chimeric HPIV vaccine viruses administered simultaneously (e.g., in a polyspecific vaccine mixture) in a primary vaccination step, e.g., at one, two or four months of age. For example, two or more and up to a full panel of HPIV-based vaccine viruses can be administered that separately express one or more antigenic determinants (i.e., whole antigens, immunogenic domains, or epitopes) selected from the G protein of RSV subgroup A, the F protein of RSV subgroup A, the G protein of RSV subgroup B, the F protein of RSV subgroup B, the HA protein of measles virus, and/or the F protein of measles virus. Coordinate booster administration of these same PIV3-based vaccine constructs can be repeated at two months of age. Subsequently, e.g., at four months of age, a separate panel of 2-6 or more antigenically distinct (referring to vector antigenic specificity) live attenuated HPIV-based vaccine viruses can be administered in a secondary vaccination step. For example, secondary vaccination may involve concurrent administration of a mixture or multiple formulations that contain(s) multiple HPIV3-1 vaccine constructs that collectively express RSV G from subgroup A, RSV F from subgroup A, RSV F from subgroup B, RSV G from subgroup B, measles virus HA, and/or measles virus F, or antigenic determinants from any combination of these proteins. This secondary immunization provides a boost in immunity to each of the heterologous RSV and measles virus proteins or antigenic determinant(s) thereof. At six months of age, a tertiary vaccination step involving administration of one-six or more separate live attenuated PIV3-2 vector-based vaccine recombinants can be coordinately administered that separately or collectively express RSV G from subgroup A, RSV F from subgroup A, RSV G from subgroup B, RSV F from subgroup B, measles virus HA, and/or measles virus F, or antigenic determinant(s) thereof. Optionally at this step in the vaccination protocol, rPIV3 and rPIV3-1 vaccines may be administered in booster formulations. In this way, the strong immunity characteristic of secondary antibody to PIV1,

PIV2, PIV3, RSV A, RSV B, and measles viruses are all induced within the first six months of infancy. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to
5 immunize against each of the three PIV viruses and other pathogens in early infancy.

In other aspects of the invention, insertion of heterologous nucleotide sequences into HPIV vaccine candidates are employed separately to modulate the level of attenuation of candidate vaccine recombinants, e.g., for the upper respiratory tract. Thus, it is possible to insert nucleotide sequences into a rHPIV that both direct the expression of a
10 foreign protein and that attenuate the virus in an animal host, or to use nucleotide insertions separately to attenuate candidate vaccine viruses. To define some of the rules that govern the effect of gene insertion on attenuation, gene units of varying lengths were inserted into a wild type HPIV3 backbone and the effects of gene unit length on attenuation were examined. These novel gene unit insertions were engineered to not contain a significant ORF which
15 permitted an evaluation of the effect of gene unit length independently of an effect of the expressed protein of that gene. These heterologous sequences were inserted as an extra gene unit of sizes between 168 nt and 3918 nt between the HN and L genes. In addition, control cDNA constructions and viruses were made in which insertions of similar sizes were placed in the 3'-noncoding region of the HN gene and hence did not involve the addition of an extra
20 gene. These viruses were made to assess the effect of an increase in the overall genome length and in gene number on attenuation. The insertion of an extra gene unit is expected to decrease the transcription of genes downstream of the insertion site which will affect both the overall abundance and ratios of the expressed proteins. As demonstrated herein, gene insertions or extensions larger than about 3000 nts in length attenuated the wild type virus
25 for the upper and lower respiratory tract of hamsters. Gene insertions of about 2000 nts in length further attenuated the rHPIV3cp45L vaccine candidate for the upper respiratory tract. In summary, gene insertions can have the dual effect of both attenuating a candidate vaccine virus and inducing a protective effect against a second virus. Gene extensions in the 3'-noncoding region of a gene, which cannot express additional proteins, can also be
30 attenuating in and of themselves. Within these methods of the invention, gene insertion length is a determinant of attenuation.

GU and NCR insertions within recombinant PIV of the invention produce an attenuation phenotype characterized by efficient replication *in vitro* and decreased

replication *in vivo*, a phenotype not previously described for other paramyxovirus insertions. The mechanism of attenuation resulting from a GU insertion may result from one or more of the following factors acting predominantly *in vivo*. The addition of an extra gene unit may decrease the level of transcription of downstream genes since there is a transcriptional

5 gradient in which more promoter-proximal genes are transcribed at a higher rate than the more promoter-distal genes. The decreased expression of the downstream gene products resulting from the decreased abundance of their mRNAs could result in attenuation if their gene product is limiting or if a specific ratio of gene products that is required for efficient replication is altered. It is thought that the transcription gradient is a consequence of the

10 transcriptase complex falling off the template during transcription as well as during the transfer across gene junctions. Alternatively, the increase in the overall length of the genome and the extra mRNAs transcribed may increase the level of viral double stranded RNA made which in turn may induce a higher level of the antiviral activity of the interferon system. Finally, the overall level of genome replication may be reduced due to the increase

15 in length of the genome and the antigenome. This may result from a disengagement of replicase complexes from the template during replication of the genomic RNA or antigenomic RNA. The decreased amount of genome available for packaging into virions may result in a decrease in virus yield which results in attenuation.

The mechanism of attenuation resulting from a NCR insertion may result

20 from one or more of the following factors. The extra length of the 3'-end of HN mRNA resulting from the NCR insertion may contribute to the instability of the mRNA and lead to a decrease in the expression of the HN protein. Alternatively, the increase in the overall length of the genome and the extra length of the HN mRNA may increase the level of viral double stranded RNA made that can induce a higher level of the antiviral activity of the

25 interferon system. Alternatively or additionally, the overall level of genome replication may be reduced due to the increase in length of the genome and the antigenome. This may result from a disengagement of replicase complexes from the template during replication of the genomic RNA or antigenomic RNA. The decreased amount of genome available for packaging into virions could result in a decrease in virus yield which results in attenuation.

30 Finally, the addition of extra nucleotides to the 3' end of the HN gene could decrease the level of transcription of downstream genes since the transcriptase complex could fall off the template during transcription of the extra nucleotides at the 3' end of the HN gene.

The *in vitro* and *in vivo* growth properties of the GU and NCR insertions into PIV3 are distinct from previous findings with other single-stranded, negative-sense RNA viruses, cited above. Previously tested insertions examined expressed proteins, whereby the independent effect of the length of insertions on viral growth *in vivo* cannot be determined.

5 The present findings demonstrate that the GU and NCR insertions greater than 3 kb specify an attenuation phenotype that is independent of expressed protein. Shorter insertions, e.g., greater than about 2 kb, specify further attenuation in a partially attenuated recipient. Also unexpectedly, the GU and NCR insertions specify restricted replication *in vivo* in the absence of restricted replication *in vitro*. In addition, the attenuation phenotype *in vivo* is
10 seen when the insertion is either in the form of a GU or a NCR insertion – other documented insertions are in the form of GU only. Thus, the attenuation of replication *in vivo* specified by a GU or NCR insertion that does not encode a protein represents a unique way to attenuate members of the Mononegavirales *in vivo*.

15 EXAMPLE I

Construction of cDNAs Encoding a Chimeric HPIV3/Measles Virus-HA Antigenome and Recovery of Infectious Virus

The full-length cDNA clones, p3/7(131)2G+, encoding the complete 15462 nucleotide antigenome of the JS PIV3 wt virus, and pFLCcp45L, which encodes the
20 antigenome of the derivative of JS wt containing three *cp45*-specific temperature-sensitive mutations in the L ORF of PIV3, have been previously described (Durbin et al., Virology 235:323-332, 1997a; Skiadopoulos et al., J. Virol. 72:1762-8, 1998, each incorporated herein by reference). These clones were used as vectors for the insertion of the HA gene of measles virus to create both wildtype and attenuated HPIV3 chimeric constructs which express a
25 heterologous antigenic determinant, exemplified by the HA protein, of measles virus. The size of each insert containing the HA gene of measles was a multiple of six such that the chimeric virus recovered from the cDNA would conform to the rule of six (Durbin et al., Virology 234:74-83, 1997b, incorporated herein by reference).

**Construction of full-length chimeric HPIV3 cDNAs encoding the HA protein of measles
30 virus in the N/P or P/M junctions.**

The *Pml*I to *Bam*HI fragment of p3/7(131)2G+ (nt 1215-3903 of the PIV3 antigenome} was subcloned into the plasmid pUC119 {pUC119(*Pml*I-*Bam*HI)} which had been modified to include a *Pml*I site in the multiple cloning region. Two independent single-stranded mutagenesis reactions were performed on pUC119(*Pml*I -*Bam*HI) using Kunkel's method (Kunkel et al., Methods Enzymol. 154:367-382, 1987, incorporated herein by reference); the first reaction introduced an *Aff*II site in the 3' (downstream)-noncoding region of the N gene by mutating the CTAAAT sequence at nts 1677-1682 of the antigenome to CTTAAG (p*Aff*II N-P), and the second, separate, reaction introduced an *Aff*II site in the in the 3'-noncoding region of the P gene by mutating the TCAATC sequence at nts 3693-3698 of the antigenome to CTTAAG (p*Aff*II P-M).

The HA ORF of measles virus Edmonston strain was amplified from Edmonston wild type virus by reverse transcription polymerase chain reaction (RT-PCR). The nt sequence of the Edmonston wild type HA open reading frame (ORF) is in GenBank Accession # U03669, incorporated herein by reference (note that this sequence is the ORF only without the upstream 3 nts or the stop codon). Measles virus RNA was purified from clarified medium using TRIzol-LS (Life Technologies, Gaithersburg, MD) following the manufacturer's recommended procedure. RT-PCR was performed with the Advantage RT-for-PCR and Advantage-HF PCR kits (Clontech, Palo Alto, CA) following the recommended protocols. Primers were used to generate a PCR fragment spanning the entire ORF of the measles virus HA gene flanked by PIV3 non-coding sequence and *Aff*II restriction sites. The forward primer 5'-
TTAATCTTAAGAATATACAAATAAGAAAACTTAGGATTAAAGAGCGATGTCAC
CACAACGAGACCGGATAAATGCCTTCTAC-3' (SEQ ID NO. 13) encodes an *Aff*II site (italicized) upstream of PIV3 noncoding sequence derived from the N/P gene junction- nts 3699-3731(underlined), containing GE, IG and GS sequences (Figure 1A) and the beginning of the measles HA ORF (bolded) preceded by three non-HPIV3, non-measles virus nts designated in the primer. The reverse primer 5'-
ATTATTGCTTAAGGTTTGTTTCGGTGTCTGTTTCTTTGTTGGATCCTATCTGCGATTG
GTTCCATCTTC-3' (SEQ ID NO. 14) encodes an *Aff*II site (italicized) downstream (in the positive-sense complement) of PIV3 noncoding sequence derived from the P gene, nt 3594-3623 (underlined), and the end of the measles HA ORF (bolded). The resultant PCR fragment was then digested with *Aff*II and cloned into p(*Aff*II N-P) and p(*Aff*II P-M) to create pUC119(HA N-P) and pUC119(HA P-M) respectively. pUC119(HA N-P) and pUC119(HA P-M) were sequenced over the entire *Aff*II insert using dRhodamine Terminator Cycle

Sequencing Ready Reaction (ABI prism, PE Applied Biosystems, Foster city, CA), and the sequence was confirmed to be correct.

The *Pml*I to *Bam*HI fragments of pUC119(HA N-P) and pUC119(HA P-M) were separately cloned into the full-length antigenome cDNA plasmid p3/7(131)2G+ as previously described (Durbin et al., *Virology* 235:323-332, 1997a, incorporated herein by reference) to create pFLC(HA N-P) and pFLC(HA P-M) (Figure 1). The *Xho*I-*Ngo*MI fragment (nt 7437-15929) of pFLC*cp45*L was then cloned into the *Xho*I-*Ngo*MI window of both pFLC(HA N-P) and pFLC(HA P-M) to create pFLC*cp45*L(HA N-P) and pFLC*cp45*L(HA P-M). pFLC*cp45*L encodes the three amino acid changes in the L gene of PIV3 *cp45* (aa position 942, 992, and 1558) which confer most of the temperature-sensitivity and attenuation of the *cp45* vaccine candidate virus (Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998, incorporated herein by reference), and the transfer of the *Xho*I-*Ngo*MI fragment transferred those mutations.

Construction of full-length HPIV3 chimeric cDNAs encoding the HA protein of measles in the HN/L junction

A HPIV3 chimeric cDNA was constructed by PCR to include a heterologous polynucleotide sequence, exemplified by the measles virus HA gene, encoding a heterologous antigenic determinant of the measles virus, flanked by the transcription signals and the noncoding regions of the HPIV3 HN gene. This cDNA was designed to be combined with an rPIV3 vector as an extra gene following the HN gene. First, using Kunkel mutagenesis (Kunkel et al., *Methods Enzymol.* 154:367-382, 1987, incorporated herein by reference), a *Stu*I site was introduced in the 3'-noncoding region of the HN gene by mutating the AGACAA sequence at nts 8598-8603 of the antigenome to AGGCCT yielding plasmid p3/7(131)2G-Stu (Figure 1B). A cDNA containing the measles HA ORF flanked by HPIV3 sequences (see Figure 1B) was then constructed in three pieces by PCR. The first PCR synthesized the left-hand, upstream piece of the gene. The forward primer 5'-
GACAATAGGCCTAAAAGGGAAATATAAAAACTTAGGAGTAAAGTTACGCAAT
CC-3' (SEQ ID NO. 15) contains a *Stu*I site (italicized) followed by HPIV3 sequence (underlined) which includes the downstream end of the HN gene (HPIV3 nts 8602-8620), an intergenic region, and the gene-start signal and sequence from the upstream end of the HN gene (HPIV3 nt 6733-6753). The reverse primer 5'-
GTAGAACGCGTTTATCCGGTCTCGTTGTGGTGACATCTCGAATTTGGATTGT

CTATTGGGTCCTTCC-3' (SEQ ID NO. 16) contains an *Mlu*I site (italicized) downstream of the start of the measles HA ORF (bolded) followed by the complement to HPIV3 nts 6744-6805 (underlined), which are part of the upstream HN noncoding region. The *Mlu*I site present in the introduced measles virus ORF was created by changing nt 27 from T (in the wild type Edmonston HA gene) to C and nt 30 from C to G. Both of these changes are noncoding in the measles virus ORF. The PCR was performed using p3/7(131)2G-Stu as template. The resulting product, termed PCR fragment 1, is flanked by a *Stu*I site at the 5'-end and an *Mlu*I site at the 3'-end and contains the first 36 nt of the measles HA ORF downstream of noncoding sequence from the HPIV3 HN gene. The second PCR reaction synthesized the right-hand end of the HN gene. The forward primer **GTAGA***ACGCGTT***TATCCGGTCTCGTTGTGGTGACAT***CTCGAATT***TGGATTGTCTATTGGGTCCTTCC**-3' (SEQ ID NO. 16) contains the *Xma*I (italics) and the end of the measles HA ORF (bold), followed by HPIV3 nts 8525-8566 (underlined) representing part of the downstream nontranslated region of the HN gene. The reverse primer 5'-CCATGTAATTGAATCCCCCAACACTAGC-3', (SEQ ID NO. 17) spans HPIV3 nts 11448-11475, located in the L gene. The template for the PCR was p3/7(131)2G-Stu. PCR fragment 2 which resulted from this reaction contains the last 35 nt of the measles HA ORF and approximately 2800 nt of the L ORF of PIV3 and is flanked by an *Xma*I site and an *Sph*I site (which occurs naturally at HPIV3 position 11317). The third PCR reaction amplified the largest, central portion of the measles HA ORF from the template cDNA pTM-7, a plasmid which contains the HA ORF of the Edmonston strain of measles virus supplied by the ATCC. Sequence analysis of this plasmid showed that the measles virus HA ORF contained in PTM-7 contains 2 amino acid differences from pTM-7 of the Edmonston wild type HA sequence used for insertion into the N-P and M-P junction, and these were at amino acid positions 46 (F to S) and at position 481 (Y to N). The forward primer 5'-CGGATAA***ACGCGTT***CTACAAAGATAACC-3' (SEQ ID NO. 18) (*Mlu*I site italicized) and reverse primer 5'-CGGATAA***ACGCGTT***CTACAAAGATAACC-3' (SEQ ID NO. 18) (*Xma*I site italicized) amplified PCR fragment 3 which contained nts 19-1838 of the measles HA ORF. To assemble the pieces, PCR fragment 1 was digested with *Stu*I and *Mlu*I while PCR fragment 3 was digested with *Mlu*I and *Xma*I. These two digested fragments were then cloned by triple ligation into the *Stu*I-*Xma*I window of pUC118 which had been modified to include a *Stu*I site in its multiple cloning region. The resultant plasmid, pUC118(HA 1+3) was digested with *Stu*I and *Xma*I while PCR fragment 2 was digested with *Xma*I and *Sph*I. The two digested products were then cloned into the *Stu*I-*Sph*I window of p3/7(131)2G-Stu.

resulting in the plasmid pFLC(HA HN-L). The *StuI-SphI* fragment, including the entire measles HA ORF, was then sequenced using dRhodamine Terminator Cycle Sequencing Ready Reaction (ABI prism, PE Applied Biosystems, Foster city, CA). The chimeric construct sequence was confirmed. In this way, the measles virus HA ORF flanked by HPIV3 transcription signals was inserted as an extra gene into the N/P, P/M, or HN/L junction of an antigenomic cDNA vector comprising a wild type HPIV3 or into the N/P or P/M junction of an antigenomic cDNA vector comprising an attenuated HPIV3.

Recovery of chimeric rPIV3 wild type and *rcp45L* expressing the HA protein of measles virus

The five full-length vector cDNAs bearing the measles HA ORF as a separate gene were transfected separately into HEp-2 cells on six-well plates (Costar, Cambridge, MA) together with the support plasmids {pTM(N), pTM(P no C), and pTM(L)}, and LipofectACE (Life Technologies), and the cells were simultaneously infected with MVA-T7, a replication-defective vaccinia virus recombinant encoding the bacteriophage T7 polymerase protein as previously described (Durbin et al., *Virology* 235:323-332, 1997; Durbin et al., *Virology* 234:74-83, 1997, each incorporated herein by reference). pTM(P no C) is a derivative of pTM(P) (Durbin et al., *Virology* 261:319-330, 1999) in which the C ORF expression has been silenced by mutation of the C start codon. After incubation at 32°C for three days, the transfection harvest was passaged onto a fresh monolayer of Vero cells in a T25 flask and incubated for 5 days at 32°C (referred to as passage 1). The presence of HPIV3 in the passage 1 harvest was determined by plaque titration on LLC-MK2 monolayer cultures with plaques visualized by immunoperoxidase staining with HPIV3 HN-specific and measles HA-specific monoclonal antibodies as previously described (Durbin et al., *Virology* 235:323-332, 1997, incorporated herein by reference).

The rPIV3(HA HN-L) virus present in the supernatant of the appropriate passage 1 harvest was biologically-cloned by plaque purification three times on LLC-MK2 cells as previously described (Hall et al., *Virus Res.* 22:173-184, 1992, incorporated herein by reference). rPIV3(HA N-P), *rcp45L*(HA N-P), rPIV3(HA P-M), and *rcp45L*(HA P-M) were biologically-cloned from their respective passage 1 harvests by terminal dilution using serial 2-fold dilutions on 96-well plates (12 wells per dilution) of Vero cell monolayers. The biologically-cloned recombinant viruses from the third round of plaque purification or from the second or third round of terminal dilution were then amplified twice in LLC-MK2 cells

{rPIV3(HA HN-L} or Vero cells {rPIV3(HA N-P), *rcp45L*(HA N-P), rPIV3(HA P-M), *rcp45L*(HA P-M)} at 32°C to produce virus for further characterization. As a first step in confirming and characterizing the recombinant chimeric PIV3s expressing the HA glycoprotein of measles virus, each passage 1 harvest was analyzed by RT-PCR using three different primer pairs; one pair for each location of the HA ORF insert. The first primer pair amplified a fragment of PIV3 spanning nucleotides 1596-1968 of the full-length HPIV3 genome, which includes the N/P insertion site. This fragment size increased to 2298 nucleotides with the measles HA ORF inserted between the N and P genes. The second primer pair amplified a fragment of PIV3 spanning nucleotides 3438-3866 of the full-length HPIV3 genome, which includes the P/M insertion site. With the measles HA ORF inserted between the P and M genes, this fragment size increased to 2352 nucleotides. The third primer pair amplified a fragment of PIV3 spanning nucleotides 8466-8649 of the full-length antigenome. With the measles HA ORF inserted between the HN and L genes, this fragment size increased to 2211 nucleotides, which includes the HN/L insertion site. All five recovered viruses contained an insert of the appropriate size at the appropriate location. The generation of each PCR product was dependent upon the inclusion of reverse transcriptase, indicating that each was derived from RNA and not from contaminating cDNA.

Monolayers of LLC-MK2 cells in T25 flasks were infected at a multiplicity of infection (MOI) of 5 with either *rcp45L*(HA N-P), *rcp45L*(HA P-M), rJS or were mock infected. Monolayers of Vero cells in T25 flasks were infected with the Edmonston wild type strain of measles virus at an MOI of 5. Vero cell monolayers were chosen for the measles Edmonston virus infection because measles virus does not grow well in LLC-MK2 cells. At 24 hours post-infection, the monolayer was washed with methionine-minus DMEM (Life Technologies). ³⁵S methionine was added to DMEM-minus media at a concentration of 10uCi/ml and 1 ml was added to each flask which was then incubated at 32°C for 6 hours. The cells were harvested and washed 3 times in PBS. The cell pellets were resuspended in 1 ml RIPA buffer {1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 (Sigma), 0.2% (w/v) SDS, 150mM NaCl, 50mM Tris-HCl, pH 7.4}, freeze-thawed and clarified by centrifugation at 6500 X G for 5 minutes. The cell extract was transferred to a fresh eppendorf tube and a mixture of monoclonal antibodies which recognizes the HA glycoprotein of measles virus (79-XV-V17, 80-III-B2, 81-1-366) (Hummel et al., J. Virol. 69:1913-6, 1995; Sheshberadaran et al., Arch. Virol. 83:251-68, 1985, each incorporated herein by reference) or which recognizes the HN protein (101/1, 403/7, 166/11) of PIV3 (van Wyke Coelingh et al., Virology 160:465-72, 1987, incorporated herein by reference)

was added to each sample and incubated with constant mixing for 2 hours at 4°C. Immune complexes were precipitated by adding 200 µl of a 10% suspension of protein A Sepharose beads (Sigma, St. Louis, MO) to each sample followed by constant mixing at 4°C overnight. Each sample was suspended in 90 µl of 1X loading buffer and 10 µl of reducing agent was added. After heating at 70°C for 10 minutes, 20 µl of each sample was loaded onto a 4-12% polyacrylamide gel (NuPAGE, Novex, San Diego, CA) per the manufacturer's recommendations. The gel was dried and autoradiographed (Figure 2). *rcp45L*(HA P-M) and *rcp45L*(HA N-P) encoded a protein precipitated by the anti-measles HA monoclonal antibodies which was the same size as the authentic measles HA protein. *rcp45L*(HA P-M) and *rcp45L*(HA N-P) expressed the measles virus HA protein to a greater extent than did the Edmonston wild type strain of measles virus indicating that these constructs efficiently expressed the measles virus HA from the N/P and P/M junctions of the attenuated strain *rcp45L*. *rcp45L*(HA N-P) and *rcp45L*(HA P-M) were confirmed to be HPIV3-based by their reactivity with the PIV3 anti-HN monoclonal antibodies.

15 **The temperature sensitivity of replication of rPIV3 parent and rPIV3(HA) chimeric viruses *in vitro***

The level of temperature sensitivity of replication of the chimeric rPIV3s bearing the measles virus HA insertion was evaluated to assess whether acquisition of the HA insert modified the level of replication in the chimeric virus compared to the parental, vector virus at various temperatures (Table 1). Serial 10-fold dilutions of *rcp45L*, *rcp45L*(N-P), *rcp45L*(HA P-M), rPIV3(HA HN-L), rPIV3(HA P-M), or rJS were carried out in L-15 supplemented with 5% FBS, 4 mM glutamine, and 50 µg/ml gentamicin on LLC-MK2 cell monolayers in 96 well plates and incubated at 32, 36, 37, 38, 39, or 40°C for 6 days. Virus was detected by hemadsorption and reported as log₁₀TCID₅₀/ml. Interestingly, chimeric derivatives of both wild type vector viruses bearing the measles virus HA gene, rPIV3(HA HN-L) and rPIV3(HA P-M), were slightly restricted in replication at 40°C (Table 1). The two attenuated rPIV3s bearing the measles virus HA gene, *rcp45L*(N-P) and *rcp45L*(HA P-M), possessed a level of temperature sensitivity similar to that of the *rcp45L* parental, vector virus with *rcp45L*(HA P-M) being slightly more ts than its parent. Thus, the viruses bearing the inserts replicated in tissue culture similarly to the parental vector rPIV3 from which they were derived, with only a slight increase in temperature sensitivity. These results indicate that rPIV3 can readily serve as a vector to accommodate the HA insert at different sites

without major alteration in replication *in vitro*, and that rPIV3(HA) chimeric viruses can readily accommodate the further addition of one or more attenuating mutations.

Table 1. Replication at permissive and elevated temperatures of recombinant HPIV3s expressing the HA protein of measles virus as an extra gene in the N-P, P-M, or HN-L junctions.

Virus	Virus titer (\log_{10} TCID ₅₀ /ml) at indicated temperature					
	32°C ¹	36°C	37°C	38°C	39°C	40°C
<i>rcp45L</i> ²	8.2	8.2	7.2	<u>5.2</u> ⁶	3.4	3.0
<i>rcp45L</i> (HA P-M) ³	7.4	6.7	<u>5.2</u>	4.2	1.4	1.4
<i>rcp45L</i> (HA N-P) ³	7.4	7.2	5.7	<u>4.2</u>	2.2	≤1.2
<i>rPIV3</i> (HA HN-L) ⁴	7.7	8.2	7.0	7.7	6.7	<u>5.2</u>
<i>rPIV3</i> (HA P-M) ⁴	7.7	7.4	6.7	6.2	6.2	<u>4.7</u>
<i>PIV3-rJS</i> ⁵	8.7	9.0	9.0	8.4	8.2	9.0

1. Permissive temperature.
2. Recombinant *ts* derivative of the JS wild type strain of HPIV3, bearing 3 attenuating amino acid substitutions derived from *cp45*.
3. Recombinant attenuated *ts* derivative of JS wild type HPIV3 expressing the HA protein of measles virus.
4. Recombinant wild type HPIV3 expressing the HA protein of measles virus.
5. Recombinant wild type HPIV3, strain JS.
6. Underlined titer represents the lowest restrictive temperature at which a 100-fold or greater reduction in titer from that at 32°C is seen and defines the shut-off temperature of the virus.

EXAMPLE II

Chimeric rPIV3s Bearing an Antigenic Determinant of Measles Virus

Replicate Efficiently in Hamsters and Induce High Titers of Antibodies Against Both HPIV3 and Measles

5

Determination of the level of replication and immunogenicity of the rPIV3(HA) viruses in hamsters

10 The levels of replication of chimeric rPIV3s bearing an antigenic determinant
of the measles virus was compared with that of their parent rPIV3s to determine if the
acquisition of the determinant, exemplified by an HA insert, significantly modified their
ability to replicate and to induce an immune response *in vivo*. In two different experiments,
groups of 6 or 7 4-6 week-old Golden Syrian hamsters were inoculated intranasally with 0.1
ml of EMEM (Life Technologies) containing $10^{6.0}$ PFU of rJS, *rcp45L*, *rcp45L*(HA P-M),
rcp45L(HA N-P), rPIV3(HA HN-L), or rPIV3(HA P-M) (Tables 2 and 3). On day 4 post-
15 inoculation the hamsters were sacrificed and the lungs and nasal turbinates were harvested.
The nasal turbinates and lungs were homogenized in 10% or 20% w/v suspension of L-15
(Quality Biologicals, Gaithersburg, MD) respectively, and the samples were rapidly frozen.
Virus present in the samples was titered on 96 well plates of LLC-MK2 cell monolayers and
incubated at 32°C for 7 days. Virus was detected by hemadsorption, and the mean
20 \log_{10} TCID₅₀/g was calculated for each group of hamsters. Insertion of the HA gene into wild
type rJS (Table 2) restricted its replication 4 to 20-fold in the upper respiratory tract and up
to five-fold in the lower respiratory tract indicating only a slight effect of the acquisition of
the HA gene on replication of wild type rJS virus in hamsters. The replication of each of the
two *rcp45*(HA) antigenic chimeras was 10-fold less in the upper respiratory tract of hamsters
25 (Table 3)-than that of *rcp45L*, the recombinant parent virus bearing the three attenuating ts
mutations in the L protein, but was the same as the *rcp45L* parent in the lower respiratory
tract. Thus, for each of the two *rcp45*(HA) antigenic chimeras there was a slight, but
statistically significant, reduction in replication in the upper respiratory tract of hamsters
indicating that the acquisition of the HA gene by *rcp45L* increased its attenuation for the
30 upper, but not the lower, respiratory tract. Thus, the effect of the insertion of the HA gene
on the replication of wild type or attenuated PIV3 was comparable in the upper respiratory
tract.

Table 2: Replication of wildtype rPIV3(HA) chimeric viruses in the upper and lower respiratory tract of hamsters

Virus ¹	# Animals	Virus Titer (log ₁₀ TCID ₅₀ /gm±S.E. ²) [Tukey-Kramer Grouping] ³	
		Nasal Turbinates	Lungs
rcp45L	8	4.0±0.1[A]	1.5±0.1[A]
rPIV3(HA N-P)	8	5.1±0.1[B]	5.9±0.1[B]
rPIV3(HA P-M)	8	5.9±0.1[C]	6.7±0.2[C]
rPIV3(HA HN-L)	8	5.9±0.2[C]	5.8±0.1[B]
rJS	8	6.5±0.1[D]	6.6±0.2[C]

1. Animals received 10⁶TCID₅₀ of the indicated virus given intranasally in a 0.1 ml inoculum and the lungs and nasal turbinates were harvested 4 days later.
2. Standard Error.
3. Mean virus titers were assigned to statistically similar groups (A-D) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and those with the same letter are not significantly different.

Table 3: Replication of the rPIV3cp45L(HA) antigenic chimeric viruses in the upper and lower respiratory tract of hamsters

Virus ¹	#Animals	Virus Titer (\log_{10} TCID ₅₀ /gm \pm S.E. ²) [Tukey-Kramer Grouping] ³	
		Nasal Turbinates	Lungs
rcp45L	6	4.7 \pm 0.2[A]	2.9 \pm 0.1[A]
rcp45L(HA N-P)	6	3.7 \pm 0.2[B]	2.9 \pm 0.1[A]
rcp45L (HA P-M)	7	3.7 \pm 0.1[B]	2.9 \pm 0.2[A]
rJS	7	6.5 \pm 0.1[C]	5.6 \pm 0.2[B]

1. Animals received 10^6 pfu of the indicated virus given intranasally in a 0.1 ml inoculum and the lungs and nasal turbinates were harvested 4 days later.
2. Standard Error.
3. Mean virus titers were assigned to statistically similar groups (A-D) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and those with the same letter are not significantly different.

The ability of the chimeric rHPIV3(HA) viruses to induce an immune response to HPIV3 and to measles virus was studied next. Groups of 6-24 Golden Syrian hamsters (age 4-6 weeks) were infected as described above with either $10^{6.0}$ PFU rJS, rPIV3(HA P-M), *rcp45L*, *rcp45L*(HA P-M), or *rcp45L*(HA N-P) (Table 4) on day 0. Serum was collected from each hamster on day -1 and on day 25 post-inoculation. The serum antibody response to HPIV3 was evaluated by hemagglutination-inhibition (HAI) assay as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985, incorporated herein by reference), and the serum antibody response to measles virus was evaluated by 60% plaque-reduction assay as previously described (Coates et al., Am. J. Epidemiol. 83:299-313, 1966, incorporated herein by reference). These results were compared with that from an additional control group of cotton rats that received $10^{5.0}$ of the live-attenuated measles virus (Moraten strain) administered intramuscularly on day 0. Cotton rats, rather than hamsters, were used in this group because measles virus is only weakly infectious for hamsters. As can be seen in Table 4, each of the PIV3(HA) chimeric viruses was able to elicit a robust serum neutralizing antibody response against measles virus. There was no significant difference between the amount of serum neutralizing antibody elicited by the attenuated derivative *rcp45L*(HA P-M) as compared to its counterpart in the wild type background, rPIV3(HA P-M). Furthermore, the level of measles virus-neutralizing serum antibodies induced by the rPIV3(HA) recombinants were on average 5-fold greater than that achieved by the intramuscular immunization with the live attenuated measles virus vaccine. In addition, the serum antibody response to HPIV3 produced by all the chimeric viruses was also robust and comparable to that produced by infection with wild type rJS.

Six hamsters from each group and from a control group similarly infected with RSV were challenged on day 25 with $10^{6.0}$ pfu of biologically-derived HPIV3 wildtype virus given intranasally in a 0.1 ml inoculum. The lungs and nasal turbinates were harvested on day 4 and processed as described above. Virus present in the samples was titrated on 96 well plates of LLC-MK2 cell monolayers and incubated at 32°C for 7 days. Virus was detected by hemadsorption and the mean \log_{10} TCID₅₀/g was calculated for each group of hamsters. As shown in Table 5, those hamsters which had received the chimeric viruses, whether in the attenuated or wild type backbone, were highly protected against replication of challenge wild type HPIV3 in both the upper and the lower respiratory tract. Thus, despite the slight attenuating effect of the acquisition of the measles virus HA gene on replication of the *rcp45*(HA) chimeric viruses, infection with either *rcp45*L(HA P-M) or *rcp45*L(HA N-P) induced a high level of protection against HPIV3 as indicated by approximately a 1000-fold reduction of its replication in the upper and lower respiratory tract of hamsters. Since wild type measles virus does not replicate efficiently in hamsters, it cannot be used to challenge this host. However, it is expected that the attenuated chimeric *rcp45*L(HA) vaccine candidates will be highly efficacious against measles virus since high levels of neutralizing antibody, i.e., mean titer of greater than 1:5000, were induced. Comparable levels of measles virus antibodies are associated with strong resistance to measles virus disease in humans (Chen et al., J. Infect. Dis. 162:1036-42, 1990, incorporated herein by reference).

Table 5. Attenuated and wildtype HPIV3-measles HA chimeric viruses are highly protective against replication of challenge wildtype PIV3 in the upper and lower respiratory tracts of hamsters.

Animals Immunized with ¹	# Animals	Virus titer (\log_{10} TCID ₅₀ /g) [Tukey-Kramer Grouping ³] Reduction in Titer (\log_{10})			
		Nasal Turbinates	Lungs	Nasal Turbinates	Lungs
RSV	6	7.0±0.3[A]	5.7±0.4[A]	NA ²	NA
rcp45L(HA P-M)	6	3.4±0.3[B]	2.9±0.0[B]	3.6	2.8
rcp45L(HA N-P)	6	2.6±0.3[B]	3.4±0.2[B]	4.4	2.3
rPIV3(HA P-M)	6	2.0±0.3[B]	3.2±0.1[B]	5.0	2.5
rcp45L	6	1.9±0.2[B,C]	3.6±0.1[B]	5.1	2.1
rJS	6	<1.4±0.0[C]	2.9±0.2[B]	>5.7	2.8

1. All groups were challenged with 10⁶ pfu biologically-derived JS wildtype PIV3 in a 0.1 ml inoculum given intranasally.
2. Not applicable.
3. Mean virus titers were assigned to statistically similar groups (A-C) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and means with the same letter are not significantly different.

EXAMPLE III

Construction of Antigenomic cDNAs Encoding a Chimeric HPIV3-1 Vector Bearing a HPIV2 HN Gene as an Extra Transcription/Translation Unit Inserted Between the F and HN Genes, and Recovery of Infectious Viruses

5 rPIV3-1 is a recombinant chimeric HPIV3 in which the HN and F genes have been replaced by those of HPIV1 (see, e.g., Skiadopoulos et al., Vaccine 18:503-510, 1999; Tao et al., Vaccine 17:1100-1108, 1999; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Patent Application Serial No. 09/458,813, filed December 10, 1999; U.S. Patent Application Serial No. 09/459,062, filed December 10, 1999, each incorporated
10 herein by reference). In the present example, the HN gene of HPIV2 was inserted into the rPIV3-1 chimeric virus that served as a vector to produce a chimeric derivative virus, bearing an introduced heterologous antigenic determinant from HPIV2, able to protect against both HPIV1 and HPIV2. The HPIV2 HN gene also was inserted into an attenuated derivative of rPIV3-1, designated rPIV3-1cp45, which contains 12 of the 15 cp45 mutations,
15 i.e., those mutations on genes other than HN and F, inserted into the rPIV3 backbone (Skiadopoulos et al., Vaccine 18:503-510, 1999). The source of the HPIV2 wild type virus was the wild type strain V9412-6 (designated PIV2/V94) (Tao et al., Vaccine 17:1100-1108, 1999), which was isolated in Vero cells from a nasal wash that was obtained in 1994 from a child with a natural HPIV2 infection. PIV2/V94 was plaque purified 3 times on Vero cells
20 before being amplified twice on Vero cells using OptiMEM tissue culture medium without FBS. A cDNA clone of the HN gene of PIV2/V94 was generated from virion RNA by reverse transcription (RT) using random hexamers and Superscript Preamplification System (Life Technologies) followed by PCR using Advantage cDNA Synthesis kit (Clontech, Palo Alto, CA) and synthetic primers which introduced *NcoI-HindIII* sites flanking the HN cDNA
25 (Figure 3A). The sequences of these primers were: (with HPIV specific sequences in upper case, restriction sites underlined, nts which are non-HPIV or which are altered from wt in lower case, and start and stop codons in bold), upstream HPIV2 HN 5'-
gggcc**ATGGA**AAGATTACAGCAAT-3' (SEQ ID NO. 19); downstream HPIV2 HN 5'-
caataagc**TTAA**AGCATTAGTTCCC-3' (SEQ ID NO. 20). The HN PCR fragment was
30 digested with *NcoI-HindIII* and cloned into pLit.PIV31HNhc to generate pLit.32HNhc (Figure 3 B). The HPIV2 HN heterologous gene insert in pLit.32HNhc was completely sequenced using the ThermoSequenase Kit and ³³P-labeled terminators (Pharmacia

Amersham, Piscataway, NJ) and was confirmed to contain the authentic sequence of the PIV2/94 HN coding region.

The HPIV2 HN gene in pLit.32HNhc was further modified by PCR and Deep Vent thermostable DNA polymerase (New England Biolab, Beverly, MA) to introduce *Ppu*MI sites for cloning into the unique *Ppu*MI site in p38'ΔPIV31hc, Figure 3C (Skiadopoulos et al., Vaccine 18:503-510, 1999). The sequences of these primers were (with HPIV specific sequences in upper case, relevant restriction sites underlined, non-HPIV nt or nt altered from wt in lower case): upstream HPIV2 HN

5'-gcgatgggcccGAGGAAGGACCCAATAGACA-3' (SEQ ID NO. 21); downstream HPIV2 HN

5'-cccgggtcctgATTTCCCGAGCACGCTTTG-3' (SEQ ID NO. 22). The modified cDNA bearing the HPIV2 HN ORF consists of (from left to right) a partial 5'-untranslated region (5'-UTR) of HPIV3 HN including the *Ppu*MI site at the 5'-end, the HPIV2 HN ORF, the 3'-UTR of HPIV3 HN, a complete set of HPIV3 transcription signals (i.e. gene stop, intergenic region and gene start sequences) whose sequences match those at the HPIV3 HN and L gene junction, a partial 5'-UTR of HPIV3 L, and an added *Ppu*MI site at its 3'-end (Figure 3C).

This fragment was digested with *Ppu*MI and inserted into p38'ΔPIV31hc digested with *Ppu*MI to generate p38'ΔPIV31hc.2HN (Figure 3D). The inserted *Ppu*MI cassette was sequenced in full and found to be as designed. The insert from p38'ΔPIV31hc.2HN was isolated as a 8.5 kb BspEI-SphI fragment and introduced into the BspEI-SphI window of pFLC.2G+.hc or pFLCcp45 to generate pFLC.31hc.2HN or pFLC.31hc.cp45.2HN, respectively (Figure 3, E and F). pFLC.2G+.hc and pFLCcp45 are full-length antigenomic clones encoding wt rPIV3-1 and rPIV3cp45, respectively, as described previously (Skiadopoulos et al., J. Virol. 73:1374-81, 1999; Tao et al., J. Virol. 72:2955-2961, 1998, each incorporated herein by reference).

Confluent HEp-2 cells were transfected with pFLC.31hc.2HN or pFLC.31hc.cp45.2HN plus the pTM(N), pTM(P no C), and pTM(L) support plasmids in the presence of MVA-T7 as previously described (Durbin et al., Virology 235:323-332, 1997, incorporated herein by reference). The recombinant chimeric viruses recovered from transfection were activated by addition of TPCK trypsin (Catalog No. 3741, Worthington Biochemical Corp., Freehold, NJ) as were all passages and titrations of viruses bearing the HPIV1 HN and F glycoproteins as described previously (Tao et al., J. Virol. 72:2955-2961,

1998, incorporated herein by reference). Recovered chimeric recombinant viruses rPIV3-1.2HN and rPIV3-1*cp*45.2HN were purified by plaque-to-plaque-to-plaque passage on LLC-MK2 monolayer in agarose overlay as previously described (Tao et al., *Vaccine* 17:1100-1108, 1999, incorporated herein by reference).

5 To determine if the rPIV3-1.2HN and rPIV3-1*cp*45.2HN recombinants contain the heterologous HPIV2 HN gene, viral RNA from each recovered recombinant chimeric virus was amplified on LLC-MK2 cells and concentrated by polyethylene glycol (PEG) precipitation (Mbiguino et al., *J. Virol. Methods* 31:161-170, 1991, incorporated herein by reference). Virion RNA (vRNA) was extracted with Trizol (Life Technologies)
10 and used as template to synthesize first strand cDNA using Superscript Preamplification system (Life Technologies, Gaithersburg, MD) and random hexamer primers as described above. The synthesized cDNA was amplified by PCR with the Advantage cDNA Synthesis kit (Clontech, Palo Alto, CA) with primers specific for HPIV1 F and HPIV1 HN coding region (for HPIV1 F 5'-AGTGGCTAATTGCATTGCATCCACAT-3' (SEQ ID NO. 23) and
15 for HPIV1 HN 5'-GCCGTCTGCATGGTGAATAGCAAT-3') (SEQ ID NO. 24). The relative locations of the PIV1 F and HN primers are indicated by arrows in Figures 3 and 4. Amplified DNA fragments were digested and analyzed on agarose gels (Figure 4). Data for rPIV3-1*cp*45.2HN is not shown, but was comparable and confirmed in structure. rPIV3-1.2HN and rPIV3-1*cp*45.2HN each contained the insert of the expected size, and the
20 digestion patterns with a number of restriction enzymes confirmed the identity and authenticity of the inserts. The presence of the *cp*45 mutations in rPIV3-1*cp*45.2HN was also confirmed.

To confirm the expression of HPIV2 HN by the rPIV3-1.2HN chimeric virus, LLC-MK2 monolayers in T25 flasks were infected with PIV2/V94, rPIV3-1, or rPIV3-
25 1.2HN at a MOI of 5 in 5 ml of serum-free OptiMEM containing 0.5 µg/ml TPCK trypsin. After incubation for 18 hours at 32°C, the flasks were washed three times with 5 ml of methionine and cysteine deficient DMEM (BioWhittaker, Walkersville, MD). Cells were then fed with 1 ml of methionine and cysteine deficient DMEM supplemented with 120 µCi of ProMix 35S-methionine and 35S-cysteine mixture (Pharmacia Amersham, Piscataway,
30 NJ) and incubated for 18 hours at 32°C. Cells were scraped into medium, pelleted by brief centrifugation in a microfuge, and washed three times with cold PBS. Each cell pellet was resuspended in 1 ml RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.2% SDS, 150 mM NaCl, and 50 mM Tris-HCl, pH7.4) containing 250 units/ml of Benzonase (Sigma),

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freeze/thawed once, and clarified by centrifugation at 12,000 X g for 5 min in a microfuge. Clarified supernatants were transferred to a clean microfuge tube, mixed with 50 µl of anti-HPIV2 HN monoclonal antibody (mAb) 150S1 (Tsurudome et al., Virology 171:38-48, 1989, incorporated herein by reference), and incubated with mixing at 4°C for 3 hours. The monoclonal antibody was precipitated by the addition to each tube of 0.2 ml of 10% Protein A sepharose suspension (in RIPA buffer) and incubation with mixing at 4° for 18 hours. The beads were washed three times with RIPA buffer and pelleted by brief centrifugation in a microfuge. Each sample was suspended in 90 µl of 1X loading buffer, and 10 µl was resolved on a 4-12% SDS polyacrylamide gel (PAGE; NOVEX, San Diego, CA). The gel was dried and autoradiographed (Figure 5). The mAb, specific to PIV2 HN, precipitated a protein from both rPIV3-1.2HN and PIV2/V94 infected LLC-MK2 cells, but not from rPIV3-1-infected cells, with a size expected for the 86kD Kd HN protein of HPIV2 (Rydbeck et al., J. Gen. Virol. 69:931-5, 1988, incorporated herein by reference).

EXAMPLE IV

The rPIV3-1 Viruses Carrying an HPIV2 Antigenic Determinant Exhibit Temperature Sensitive Phenotypes Similar to Those of Their Parental Vector Viruses

The level of temperature sensitivity of replication of rPIV3-1.2HN and rPIV3-1.*cp45*.2HN in LLC-MK2 cells was evaluated to determine if the acquisition of the HN ORF of HPIV2 by rPIV3-1 wild type or attenuated viruses employed as vectors altered the level of temperature sensitivity of replication in the resultant chimeric derivatives bearing the heterologous antigenic determinant of HPIV2 compared to the parental, vector viruses (Table 6). rPIV3-1.2HN and rPIV3-1*cp45*.2HN, along with control viruses, were serially diluted 1:10 in 1X L15 supplemented with 0.5 µg/ml TPCK trypsin and used to infect LLC-MK2 monolayers in 96 well plates in quadruplicate. Infected plates were placed at various temperatures for 7 days before the virus titers were determined by hemadsorption using 0.2% guinea pig erythrocytes (in 1X PBS). The virus titers are presented as log₁₀TCID₅₀ ± standard error (S.E.). As shown in Table 6, rPIV3-1.2HN and rPIV3-1*cp45*.2HN exhibited a level of temperature sensitivity similar to that of their parental, vector viruses, i.e. rPIV3-1 and rPIV3-1*cp45*, respectively, each of which lacks the HPIV2 HN insert. This indicated that the introduction of one extra transcription/translation unit in rPIV3-1.2HN and rPIV3-

1cp45.2HN, does not significantly alter their level of temperature sensitivity of replication *in vitro*.

Table 6. The rPIV3-1 viruses carrying the PIV2 HN insertion have a temperature sensitive phenotype similar to that of their parental virus.

Virus	Titer at 32° C ^a (log ₁₀ TCID ₅₀)		Titer reduction (log ₁₀ TCID ₅₀) at various temperatures (°C) ^a					
			35° ^b	36°	37°	38°	39°	40°
PIV2/V9412	7.8	0.3	(0.1) ^c	0.0	(0.4)	(0.4)	0.0	
PIV1/Wash64	8.5	1.5	1.1	1.4	0.6	0.5	0.9	
rPIV3/JS	7.9	0.3	0.1	0.1	(0.3)	(0.4)	0.4	
PIV3 <i>cp45</i>	7.8	0.5	0.3	1.3	<u>3.4</u> ^d	6.8	6.9	
rPIV3-1	8.0	0.8	0.5	0.6	0.9	1.1	<u>2.6</u>	
rPIV3-1.2HN	8.3	0.5	(0.3)	0.3	0.6	1.5	<u>2.6</u>	
rPIV3-1 <i>cp45</i>	8.0	0.5	0.4	<u>3.4</u>	4.8	6.6	7.5	
rPIV3-1 <i>cp45.2HN</i>	8.0	0.3	1.4	<u>2.9</u>	5.3	7.6	7.6	

^a Data presented are means of two experiments.

^b Data at 35°C were from single experiment.

^c Numbers in parentheses represent titer increase.

^d Underlined value indicates shut-off temperature at which the virus titer showed a reduction of 100-fold or more in comparison to the titer at 32°C.

EXAMPLE V

Replication and Immunogenicity of rHPIV3-1.2HN Chimeric Viruses in Animals

To determine the level of replication of the chimeric viruses *in vivo*, Golden Syrian hamsters in groups of six were inoculated intranasally with 0.1 ml of 1X L-15 medium containing $10^{5.3}$ TCID₅₀ (or 10^6 pfu) of virus (Table 7). Four days after infection, hamsters were sacrificed and their lungs and nasal turbinates harvested. Virus titers, expressed as mean log₁₀TCID₅₀/gram of tissue (Table 7), were determined. rPIV3-1 expressing the PIV2 HN gene, termed rPIV2-1.2HN, is more restricted in replication than its rPIV3-1 parent as indicated by a 30-fold reduction in virus titer in both the upper and lower respiratory tracts of hamsters. Thus, the insertion of a transcription/translation unit expressing the PIV2 HN protein into rPIV3-1 attenuates the virus for hamsters. The attenuating effect of insertion of a transcription/translation unit containing PIV2 HN ORF into rPIV3-1 was slightly more than that observed for the insertion of a similar unit containing the measles HA ORF into the recombinant JS strain of wild type PIV3. The rPIV3-1*cp45*.2HN virus was 1,000-fold more restricted in replication than the rPIV3-1*cp45* parent indicating that the attenuating effect of the PIV2 HN insertion and the *cp45* mutations are additive. It should be possible to adjust the level of attenuation as needed by adding fewer *cp45* mutations than the 12 that are present in rPIV3-1.*cp45*.2HN.

Table 7. The chimeric rPIV3-1 expressing the HN glycoprotein of PIV2 (rPIV3-1.2HN) is attenuated in the respiratory tract of hamsters

Experiment No.	Virus	Virus titer in indicated tissue log ₁₀ TCID ₅₀ /g±S.E.) ^c	
		NT	Lungs
1 ^a	rPIV3-1	6.9 ± 0.1[A] ^d	6.0 ± 0.3[A]
	rPIV3-1.2HN	5.4 ± 0.2[B]	4.4 ± 0.4[C]
2 ^b	rPIV3-1	6.7 ± 0.1[A]	6.6 ± 0.2[A]
	rPIV3-1.2HN	5.1 ± 0.1[B, C]	5.2 ± 0.2[B]
	rPIV3-1cp45	4.6 ± 0.3[C]	1.8 ± 0.4[D]
	rPIV3-1cp45.2HN	1.5 ± 0.1[D]	≤1.2[D]
	rPIV3/JS	6.5 ± 0.2[A]	6.7 ± 0.1[A]
	rcp45	4.9 ± 0.2[B, C]	1.2 ± 0.04[D]

^a Groups of six animals were inoculated intranasally with 10⁶ pfu of indicated virus in 0.1 ml medium on day 0.

^b Groups of 6 hamsters were inoculated intranasally as in Experiment 1 with 10^{5.3} TCID₅₀ of indicated virus on day 0.

^c Lungs and nasal turbinates of the hamsters were harvested on day 4. Virus titers in tissue were determined and the titer expressed as log₁₀TCID₅₀/gram ± standard error (S.E.). NT = nasal turbinates.

^dMeans in each column with a different letter are significantly different (α=0.05) by Duncan's Multiple Range test whereas those with the same letter are not significantly different.

Since the single rPIV3-1.2HN virus expresses protective antigens of PIV1 (the F and HN glycoprotein) and PIV2 (the HN glycoprotein only), infection with this virus will induce resistance against challenge with either PIV1 or PIV2 wild type viruses. To verify this, Golden Syrian hamsters in groups of 12 were immunized intranasally with $10^{5.3}$

- 5 TCID₅₀ of virus as described above. Half of the hamsters were challenged with PIV2 on day 29, the remaining half with PIV1 on day 32. Hamster lung and nasal turbinate tissues were harvested 4 days after challenge, and titer of challenge virus were determined as described above (Table 8). Sera were obtained before and 28 days after immunization and tested for their neutralizing antibody titer against PIV1 and PIV2.

Table 8. The chimeric rPIV3-1 virus expressing the HN glycoprotein of PIV2 (rPIV3-1.2HN) protects hamsters against challenge with both PIV1 and PIV2

Immunizing virus ^a	Serum neutralizing antibody titer against indicated virus (reciprocal mean log ₂ ±SE) ^b				Titer of challenge virus in indicated tissues (log ₁₀ TCID ₅₀ /g±SE) ^c			
	PIV1		PIV2		PIV1		PIV2	
	pre	post	pre	post	NT	Lung	NT	Lung
rPIV3/JS	≤4.0±0.0	≤4.0±0.0	4.5±0.1	4.6±0.2	5.4±0.2	5.1±0.1	6.8±0.2	6.0±0.3
PIV2	≤4.0±0.0	≤4.0±0.0	4.3±0.2	9.6±0.2	5.7±0.2	5.7±0.2	≤1.2	≤1.2
rPIV3-1	4.2±0.1	8.5±0.3	4.0±0.0	4.2±0.1	≤1.2	≤1.2	6.3±0.1	6.5±0.2
rPIV3-1.2HN	≤4.0±0.0	6.2±0.2	4.1±0.1	8.3±0.2	2.3±0.5	≤1.2	≤1.2	≤1.2
rPIV3-1cp45	≤4.0±0.0	6.2±0.4	≤4.0±0.0	4.0±0.0	3.6±0.3	2.7±0.5	6.0±0.1	5.7±0.4
rPIV3-1cp45.2HN	4.0±0.9	4.1±0.1	4.0±0.0	4.2±0.1	5.1±0.2	4.8±0.2	6.8±0.1	6.6±0.2

^a Hamsters in groups of 12 were immunized with 10^{5.3} TCID₅₀ of indicated virus intranasally on day 0.

^b Serum was diluted 1:10 with OptiMEM and heat-inactivated by incubation at 56° for 30 min. The serum neutralizing antibody titer was determined on LLC-MK2, and the titers are expressed as reciprocal mean log₂±standard error (SE).

^c Half of the hamsters from each immunized group were challenged with 10⁶ TCID₅₀ PIV2 on day 29, and the remaining half were challenged with 10⁶ TCID₅₀ PIV1 on day 32. Tissue samples were harvested 4 days after challenge, and challenge virus titers are expressed as log₁₀ TCID₅₀/gram of tissue ± SE. NT = nasal turbinates.

were introduced into the downstream noncoding region (NCR) of the HN gene. Thus, two series of rPIV3s were made containing insertions of increasing length: in the GU series, the insert was added as an extra gene encoding an extra mRNA, while in the NCR series the insert was made so that the gene number was unchanged.

5 Construction of cDNAs encoding rHPIV3 viruses containing GU and 3'-NCR insertions

Insertion mutations were constructed in a pUC based plasmid, pUC118-Stu, containing the *Xho*I to *Sph*I fragment (HPIV3 nts 7437-11317) of the full length HPIV3 clone p3/7(131)2G-Stu. Two separate plasmids were constructed as acceptor plasmids for insertion of GUs and HN gene 3'-NCR extensions (Figure 6). In each, a synthetic

10 oligonucleotide duplex containing multiple cloning sites was inserted into the unique *Stu* I site. The inserted sequence for the GU insertion plasmid contained a HN gene-end (GE) signal sequence, the conserved intergenic (IG) trinucleotide sequence, and a L gene-start (GS) signal sequence, cis-acting sequences that direct termination of the HN gene transcription and initiation of transcription of the inserted sequence, respectively (Figure 6).

15 Additional unique restriction endonuclease sites were included in the multiple cloning region to facilitate subsequent screening and subcloning. The 3'-NCR extension acceptor plasmid was similarly designed and constructed, but it lacked the cis-acting GE, IG, and GS sequences at its 5'-end (Figure 6B, Table 9). The RSV antigenomic plasmid d53RSV sites or subgenomic plasmid pUC118FM2 (Table 9) were digested with the appropriate restriction

20 enzymes, and fragments of the desired sizes were isolated by electrophoresis on agarose gels and ligated individually into the unique *Hpa*I site of the GU or the HN gene 3'-NCR extension acceptor plasmid (Figure 6; Table 9). Clones were screened to identify ones in which the RSV restriction fragments were inserted in the reverse orientation, an orientation in which all reading frames contained multiple stop codons (Figure 7). Short synthetic

25 oligonucleotide duplexes ranging in size from 13 to 17 nucleotides also were inserted as necessary into the GU or 3'-NCR acceptor plasmids to modify the genome length to conform to the "rule of six" (Table 9). The specific RSV sequences and size of the short synthetic oligonucleotides added are summarized in Table 9. Plasmid clones were sequenced through all restriction enzyme sites used for subcloning, and *Xho*I-*Sph*I fragments containing

30 insertion mutations conforming to the rule of six, either as GUs or HN gene NCR extensions, were cloned into the full-length PIV3 cDNA plasmid p3/7(131)2G+. One insert, containing the 1908 GU insert, also was placed into an antigenomic cDNA bearing the three L mutations of *cp45*.

Table 9: Sources of nucleotides used to create the gene unit (GU) and HN gene 3' non coding region (NCR) extension insertions.

Restriction fragment size (nts)	Restriction sites and nt position in the RSV antigenome	GU multiple cloning site (58 nt) + rule of 6 oligonucleotide ^e	GU insertion (total nts inserted)	NCR multiple cloning site (32 nt) + rule 6 oligonucleotide ^e	NCR insertion (total nts inserted)
97 ^a	<i>SspI</i> - <i>SspI</i> ; 7272-7369	+58 + 13	168	nd	nd
212 ^b	<i>HpaI</i> - <i>HpaI</i> ; 12243-12455	nd	nd	+32 + 14	258
603 ^b	<i>SspI</i> - <i>SspI</i> ; 309-912	+58 + 17	678	nd	nd
925 ^b	<i>HpaI</i> - <i>HpaI</i> ; 12455-13380	+58 + 13	996	+32 + 15	972
1356 ^{b,c}	<i>HincII</i> - <i>HincII</i> ; 5060-6417	+58 + 14	1428	+32 + 16	1404
1850 ^{b,d}	<i>HpaI</i> - <i>HpaI</i> ; 12455-13380	+58 + 0	1908	nd	nd
3079 ^b	<i>EcoRV</i> - <i>EcoRV</i> ; 1403-4482	nd	nd	+32 + 15	3126
3845 ^b	<i>ScaI</i> - <i>ScaI</i> ; 344-4189	+58 + 15	3918	+32 + 17	3894

a. Source of RSV sequence is pUC118FM2, a plasmid containing a subgenomic cDNA fragment of RSV subgroup A as described previously (Juhász, K. et al, J Virol., 71:5814-5819, 1997.). b. Source of RSV sequence is D53sites, a plasmid containing the entire RSV subgroup A cDNA sequence with several introduced point mutations as described previously. The previously described D53sites plasmid was used to derive the rAsites virus described in Whitehead, S. et al. J. Virol., 72:4467-4471, 1998. c. The gel purified 1356 nt fragment contained a 1 nt deletion compared to the predicted 1357 nt restriction endonuclease cleavage product. d. The 1850 nt fragment is a product of two 3' to 3' adjointed 925 nt restriction fragments. e. The following oligonucleotides were inserted into the *MluI* restriction site to conform all the inserted foreign sequences to the rule of six: 13mer: CGCGGCGAGGCCTG (SEQ ID NO. 25); 14mer: CGCGGCGAGGCCTG (SEQ ID NO. 26); 15mer: CGCGAGGCCTCCGCG (SEQ ID NO. 27); 16mer: CGCGCGCGGAGGCCT (SEQ ID NO. 28); 17mer: CGCGCGCGGAGGCCT (SEQ ID NO. 29). nd, not done.

Recovery of recombinant PIV3s bearing insertion mutations

Full-length antigenomic cDNA derivatives bearing the insertion mutations and three support plasmids pTM(N), pTM(P no C) and pTM(L) (Durbin et al., *Virology* 235:323-332, 1997; Durbin et al., *Virology* 261:319-330, 1999, each incorporated herein by reference) were transfected into HEp-2 monolayers in 6-well plates (Costar, MA) using LipofectACE (Life Technologies, MD), and the monolayers were infected with MVA-T7 as described previously (Durbin et al., *Virology* 235:323-332, 1997; Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998, each incorporated herein by reference). After incubation at 32°C for 4 days, the transfection harvest was passaged onto LLC-MK2 cells in T-25 flasks which were incubated at 32°C for four to eight days. The clarified medium supernatant was subjected to plaque purification on LLC-MK2 cells as described previously (Durbin et al., *Virology* 235:323-332, 1997; Hall et al., *Virus Res.* 22:173-184, 1992; Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998, each incorporated herein by reference). Each biologically-cloned recombinant virus was amplified twice in LLC-MK2 cells at 32°C to produce virus for further characterization. Virus was concentrated from clarified medium by polyethylene glycol precipitation (Mbiguino et al., *J. Virol. Methods* 31:161-170, 1991, incorporated herein by reference), and viral RNA (vRNA) was extracted with Trizol Reagent (Life Technologies). Reverse transcription was performed on vRNA using the Superscript II Preamplification System (Life Technologies) with random hexamer primers. The Advantage cDNA PCR kit (Clontech, CA) and sense (PIV3 nt 7108-7137) and antisense primers (PIV3 nt 10605-10576) were used to amplify fragments for restriction endonuclease digestion or sequence analysis. The PCR fragments were analyzed by agarose gel electrophoresis (Figure 8) and sequencing. Each of the recovered rPIV3 insertion mutants contained insertions of the indicated sizes and they were next evaluated for their biological properties.

EXAMPLE VII

Replication of rHPIV3 Viruses Containing GU or NCR Inserts in Animals and in Tissue Culture

Multi-step growth curves

The growth properties of the rPIV3 GU and NCR insertion mutants were compared to rPIV3 wt and *rcp45_L* *in vitro*. As shown in Figure 9, the rate of replication and the peak virus titer of each of the rPIV3s containing either the GU or NCR insertions was

indistinguishable from that of rPIV3 wt indicating that insertion of sequences of at least 3918 nts in length does not affect virus replication *in vitro*.

Replication in hamsters of rPIVs containing GU insertions

- Hamsters were inoculated intranasally with $10^{6.0}$ TCID₅₀ rPIV3wt, *rcp45_L* or
- 5 with one of the indicated mutant rPIV3s bearing GU insertions (Table 10). Lungs and nasal turbinates were harvested on day four after infection and the level of replication of each virus was determined. Insertion of GUs ranging in size from 168 nt up to 1908 nt did not significantly reduce viral replication in the respiratory tract of hamsters. However, insertion
- 10 of a 3918 nt gene unit between the HN and L ORF of wild type PIV3 resulted in a 5 and 25-fold reduction in viral replication in the nasal turbinates and lungs, respectively. This indicates that gene unit insertions of this size are attenuating for a wild type virus whereas shorter sizes, e.g., below approximately 2000 nt, have little effect on replication of wild type virus in the respiratory tract of hamsters. Thus, GU length can be altered to determine a desired level of attenuation in PIV vaccine viruses.

Table 10: Replication of rPIV3 GU insertion mutants in the respiratory tract of hamsters

Virus ^a	Mean virus titer (\log_{10} TCID ₅₀ /g \pm S.E. ^b) in:	
	Nasal Turbinates	Lungs
rPIV3 wt	5.9 \pm 0.2	6.0 \pm 0.2
r168 nt GU ins	5.9 \pm 0.1	6.4 \pm 0.1
r678 nt GU ins	6.1 \pm 0.1	6.2 \pm 0.1
r996 nt GU ins	5.5 \pm 0.2	5.4 \pm 0.2
r1428 nt GU ins	5.9 \pm 0.1	5.3 \pm 0.6
r1908 nt GU ins	5.6 \pm 0.1	5.7 \pm 0.2
r3918 nt GU ins	5.2 \pm 0.2	4.6 \pm 0.3
rcp45 _L	3.1 \pm 0.0	1.7 \pm 0.2
r1908 nt GU ins/cp45 _L	1.8 \pm 0.2	1.5 \pm 0

a. Hamsters, in groups of eight, were administered $10^{6.0}$ TCID₅₀ of virus intranasally in a 0.1 ml inoculum. Lungs and nasal turbinates were harvested four days later and virus titer was determined at 32°C. **b.** S.E.: Standard error.

As described above, the insertion of the HA gene of measles virus into the rJS wildtype and the attenuated *cp45L* virus further attenuated each virus for hamsters. Since the HA gene of measles virus is 1936 nt in length, we examined the effect of a similar size gene insertion (1908 nt) on replication of *rcp45L*. The 1908 gene insertion differs from the measles virus HA gene insertion in that it cannot synthesize a large polypeptide. When the 1908 nt GU insertion was combined with the *cp45L* polymerase amino acid substitutions (r1908 nt GU ins/*cp45L* in Table 10), attenuation was augmented approximately 20-fold in the upper respiratory tract. Considered together, these findings indicate that GU insertions of approximately 3918 nts in length can attenuate a wild type PIV3 virus for hamsters and that GU insertions of about half this size can further attenuate an attenuated PIV3 vaccine candidate. Thus, GU insertions can have dual roles in the design of recombinant vaccines. The first role is to encode a protective antigen of a pathogen, and the second role is to confer an attenuation phenotype.

Replication in hamsters of rPIVs containing HN gene 3'-NCR insertions.

Hamsters were inoculated intranasally with rPIV3 control viruses or viruses bearing insertion mutations extending the length of the HN gene 3'-NCR (Table 11). Lungs and nasal turbinates were harvested four days after inoculation and the level of viral replication in each tissue was determined as described above. HN gene NCR insertions ranging in size from 258 nt up to 1404 nt did not significantly reduce viral replication in the respiratory tract of hamsters (Table 3). However, an insertion of 3126 nt effected a 16-fold reduction in viral titer in the upper and lower respiratory tracts of infected hamsters, and a 3894 nt HN gene NCR insertion resulted in a 12-fold reduction of viral replication in the upper and lower respiratory tracts, suggesting that increasing the genome length also confers an attenuating effect on viral replication.

Table 11: Replication of rPIV3 NCR insertion mutants in the respiratory tract of hamsters

Virus ^a	Mean virus titer (\log_{10} TCID ₅₀ /g \pm S.E. ^b) in:	
	Nasal Turbinates	Lungs
rPIV3 wt	6.2 \pm 0.1	6.4 \pm 0.1
r258 nt NCR ins	5.9 \pm 0.1	6.5 \pm 0.1
r972 nt NCR ins	5.9 \pm 0.1	6.6 \pm 0.1
r1404 nt NCR ins	5.9 \pm 0.2	6.6 \pm 0.1
r3126 nt NCR ins	5.0 \pm 0.1	5.2 \pm 0.1
r3894 nt NCR ins	5.1 \pm 0.1	5.3 \pm 0.1
rcp45 _L	3.4 \pm 0.1	1.9 \pm 0.2

a. Hamsters, in groups of eight, were administered $10^{6.0}$ TCID₅₀ of virus intranasally in a 0.1 ml inoculum. Lungs and nasal turbinates were harvested four days later and virus titer was determined at 32°C. **b.** S.E.: Standard error.

Evaluation of the level of temperature sensitivity of GU and NCR insertions

The efficiency of plaquing (EOP) at permissive and non-permissive temperatures of rPIVs was determined on LLC-MK2 monolayers as described above (Table 12). At 32°C, viruses bearing GU insertions ranging in size from 168 nt up to 3918 nt and NCR insertions ranging in size from 258 nt up to 3894 nt had a plaque morphology that was similar to that of rPIV3 wt. However, at 39°C and at higher temperatures all of the viruses bearing insertion mutations had a small plaque phenotype (Table 12). The GU insertions ranging in size from 996 nt up to 3918 nt yielded viruses that were not ts at 40°C. However, viruses bearing HN gene NCR insertions of 1404 nts or greater yielded viruses that were slightly ts at 40°C with a gradient of temperature sensitivity proportional to the size of the insertion. Addition of the 1908 nt GU insertion to the *cp45_L* backbone yielded a virus that was almost 100-fold more ts at 38°C compared to *rcp45_L*, demonstrating that the ts phenotype specified by the 1908 nt GU insertion and by the L gene *ts* mutations is additive.

Table 12: Efficiency of plaque formation of rPIV3 GU and NCR insertion mutants at permissive and non-permissive temperatures

Virus	Virus titer at indicated temperature (log ₁₀ PFU/ml)				
	32°C	37°C	38°C	39°C	40°C
rPIV3 wt	7.8	ND	ND	7.4	7.5
r168 nt GU ins	7.8	ND	ND	7.5 ^a	6.7 ^a
r678 nt GU ins	7.9	ND	ND	7.3 ^a	7.0 ^a
r996 nt GU ins	7.7	ND	ND	7.0 ^a	6.3 ^a
r1428 nt GU ins	7.8	ND	ND	7.4 ^a	6.4 ^a
r1908 nt GU ins	7.6	ND	ND	6.5 ^a	6.0 ^a
r3918 nt GU ins	6.3	ND	ND	5.7 ^a	5.0 ^a
r258 nt NCR ins	8.1	ND	ND	7.4 ^a	7.5 ^a
r972 nt NCR ins	8.2	ND	ND	7.8 ^a	7.8 ^a
r1404 nt NCR ins	6.7	ND	ND	5.2 ^a	<u><3.7</u>
r3126 nt NCR ins	7.4	ND	ND	6.4 ^a	<u>4.5^a</u>
r3894 nt NCR ins	7.4	ND	ND	<u>5.3^a</u>	5.0 ^a
rcp45 _L	7.8	7.3	6.0	<u><0.7</u>	ND
r1908 nt GU ins/cp45 _L	6.7	5.0 ^a	<u>3.0^a</u>	<u><0.7</u>	ND
rcp45	8.1	6.7	<u>5.7^a</u>	2.0 ^a	ND

a. Plaques were enumerated by immunoperoxidase staining after incubation for 6 days at the indicated temperature. Values which are underlined and in bold type represent the lowest restrictive temperature at which there was at least a 100-fold reduction of plaque efficiency compared to the titer at 32°C, which is defined as the shut-off temperature of plaque formation.

Since the r3918 nt GU insertion mutant as well as the r3126 nt and r3894 nt NCR insertion mutants replicated efficiently *in vitro* but were restricted in replication in the respiratory tract of hamsters, these recombinants exhibit a novel, host-range attenuation phenotype.

Based on the foregoing examples, it is demonstrated that recombinant HPIV3 (rHPIV3) provides an effective vector for foreign viral protective antigens expressed as additional, supernumerary genes, as exemplified by the measles virus hemagglutinin (HA) glycoprotein gene. In another embodiment, the rHPIV3-1 antigenic chimeric virus, a recombinant HPIV3 in which the PIV3 F and HN genes were replaced by their HPIV1 counterparts, provides an effective vector the HPIV2 hemagglutinin-neuraminidase (HN) glycoprotein. In each case, the foreign coding sequence was designed and constructed to be under the control of a set of HPIV3 gene start and gene end transcription signals, inserted into the vector genome as an additional, supernumerary gene, and expressed as a separate mRNA by the HPIV3 polymerase.

Expression of the measles virus HA or the HPIV2 HN glycoprotein from a supernumerary gene insert by the rHPIV3 or rHPIV3-1 vector was determined to be stable over multiple rounds of replication. Hamsters infected with the rHPIV3 vector expressing the measles virus HA or the rHPIV3-1 vector expressing the HPIV2 HN glycoprotein induced a protective immune response to HPIV3 and measles virus, or to HPIV1 and HPIV2, respectively. Thus, a single rHPIV3 vector expressing the protective antigen of measles virus induced a protective immune response against two human pathogens, namely, HPIV3 via an immune response to the glycoproteins present in the vector backbone and measles virus via the HA protective antigen expressed from the extra gene inserted into rHPIV3. The measles virus glycoprotein was not incorporated into the infectious HPIV3 vector virus, and hence its expression would not be expected to alter the tropism of the vector nor render it susceptible to neutralization with measles virus-specific antibodies. Similarly, a single rHPIV3-1 vector expressing the protective HN antigen of HPIV2 induced a protective immune response against two human pathogens, namely, HPIV1 via an immune response to the glycoproteins present in the vector backbone and HPIV2 via the HN protective antigen expressed from the extra gene inserted into rHPIV3-1.

EXAMPLE VIII

A Single rHPIV3 Expressing Up To Three Supernumerary Foreign Viral Glycoproteins Induces Protective Antibodies Against Up To Three Viruses

5 Modification of a single recombinant vaccine virus to induce immunity
against multiple pathogens has several advantages. It is much more feasible and expeditious
to develop a single attenuated backbone expressing antigens against multiple pathogens than
it is to develop a separate attenuated vaccine against each pathogen. Each pathogen offers
different challenges for manipulation, attenuation and demonstration of safety and efficacy,
10 and it would be a daunting task to attempt to develop an attenuated version of each of a
series of pathogens. It is also simpler and easier to prepare, handle, and administer a single
vaccine virus than to undertake these activities with several different attenuated viruses.
Reducing the number of vaccine viruses also will help simplify the crowded schedule of
pediatric immunizations. Several attenuated viruses can be administered as a mixture, but
15 this complicates vaccine development, since each component must be shown to be safe
separately, and then shown to be safe and efficacious as a mixture. One particular problem
with the administration of mixtures of viruses is the common phenomenon of viral
interference, in which one or more of the viruses in the mixture interferes with the
replication of one or more of the other components. This may result in reduced replication
20 and immunogenicity for one or more components. This common problem is obviated by the
use of a single vector backbone. Also, since some viruses such as measles virus have
particular safety concerns, it would be safer to use a single, comparatively benign virus such
as PIV as a vector bearing multiple supernumerary antigens, as opposed to a mixture of
separately-attenuated viruses, each of which must be developed and validated separately.

25 In the present example recombinant HPIVs are constructed and shown to
serve as vectors for more than one supernumerary gene with satisfactory characteristics of
replication and immunogenicity for development of vaccine viruses. In particular, this
example describes the design, construction, recovery, and characterization of rHPIV3s
expressing one, two or three supernumerary genes from the following list: (i) the
30 hemagglutinin-neuraminidase (HN) of HPIV1 (Washington/20993/1964 strain); (ii) the HN
of HPIV2 (V9412 strain); (iii) the hemagglutinin (HA) of the wild type Edmonston strain of
measles virus; and (iv) a 3918-nt translationally-silent synthetic gene called gene unit (GU)
(see above). The added genes were inserted into rHPIV3 between the nucleoprotein (N) and

phosphoprotein (P) genes, between the P and membrane protein (M) genes, or between the HN and large polymerase (L) genes. Thus, the disclosure demonstrates the successful use of an HPIV3 vector modified into a bivalent, trivalent, or quadrivalent vaccine recombinant capable of inducing multivalent immunity, e.g., against the vector itself and one or two additional pathogens.

Insertion of the HPIV1 HN and HPIV2 HN genes between the N/P and P/M genes was performed as follows: Plasmid pUC119(*Afl*III N-P), a subclone of the HPIV3 antigenomic cDNA (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference), was modified by site directed mutagenesis to insert a unique *Afl*III site into (i) the downstream noncoding region of the HPIV3 N gene (CTAAAT to CTTAAG, HPIV3 nts 1677-1682), or (ii) the downstream noncoding region of the HPIV3 P gene (TCAATC to CTTAAG, HPIV3 nts 3693-3698). Each *Afl*III site was then modified by the insertion of an oligonucleotide duplex, creating the intermediate plasmids pUC(GE/GS-N-H)_{N-P} and pUC(GE/GS-N-H)_{P-M}, respectively. The inserted duplex contained an HPIV3 gene-end (GE) sequence, the conserved intergenic (IG) trinucleotide sequence, and an HPIV3 gene-start (GS) sequence, which are cis-acting signals that direct transcriptional termination and initiation, respectively (Figure 10). Additional unique restriction endonuclease sites were included in the multiple cloning region to facilitate subsequent subcloning and screening, including *Nco*I and *Hind*III sites for addition of the HPIV1 and HPIV2 HN ORFs. Thus, a foreign ORF inserted into the multiple cloning site would be under the control of a set of HPIV3 transcription signals and expressed as a separate mRNA by the HPIV3 polymerase. The multiple cloning site also contained an *Mlu* I site for inserting oligonucleotides of varying lengths as necessary to make the entire inserted sequence conform to the rule of six (Calain et al., *J. Virol.* 67:4822-30, 1993; Durbin et al., *Virology* 234:74-83, 1997b; 1999a Skiadopoulos et al., *Virology* 272:225-34, 2000).

The HPIV1 HN ORF, available as an *Nco*I to *Hind*III restriction fragment of p38'Δ31hc #6 (Tao et al., *J. Virol.* 72:2955-2961, 1998), was inserted into the *Nco*I to *Hind*III sites of pUC(GE/GS-N-H)_{N-P} and pUC(GE/GS-N-H)_{P-M} to generate pUC 1HN_{N-P} and pUC 1HN_{P-M}, respectively. Short oligonucleotide duplexes were inserted in the unique *Mlu*I restriction site to adjust the sequence to conform to the rule of six. These chimeric subgenomic cDNAs were then cloned into the full-length HPIV3 antigenomic cDNA p3/7(131)2G⁺, referred to here as pFLC HPIV3 wt, to yield pFLC HPIV3 1HN_{N-P} and pFLC

HPIV3 1HN_{P-M}, respectively (Figure 11, the plasmids from which the second and third recombinant viruses from the top were isolated).

The HPIV2 HN ORF, available within an *Nco*I to *Hind*III restriction fragment of p32Hnhc#3 31hc (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), was inserted into the *Nco*I to *Hind*III sites of pUC(GE/GS-H-N)_{N-P} and pUC(GE/GS-H-N)_{P-M} to generate pUC 2HN_{N-P} and pUC 2HN_{P-M}, respectively. Short oligonucleotide duplexes were inserted in the unique *Mlu*I restriction site to adjust the sequence to conform to the rule of six. Inadvertently, the inserted oligonucleotide was one nucleotide shorter than that required to specify that the genome of the recovered virus would conform to the rule of six. Therefore, all cDNAs bearing the HIV2 HN gene insertion did not conform to the rule of six. Nonetheless, virus was recovered from each of these cDNAs. These chimeric subgenomic cDNAs were cloned into the full-length PIV3 antigenomic cDNA pFLC HPIV3 wt to yield pFLC PIV3 2HN_(N-P) and pFLC PIV3 2HN_(P-M), respectively (Figure 11, plasmids from which the fourth and fifth recombinant viruses from the top were isolated).

Additional recombinant HPIV3 antigenomic cDNAs were assembled that contained up to three supernumerary foreign genes in various combinations and locations in the HPIV3 backbone (Figure 11). These antigenomic cDNAs were assembled from the subgenomic cDNAs described above in which the HN of HPIV1 or HPIV2 was inserted between the N and P genes or the P and M genes. Other subclones used for assembly contained the measles virus HA gene between the P/M genes or HN/L genes as described above. Another subclone used in assembly contained the 3918-nt GU between the HN and L genes, as described above.

The recombinants containing two or three supernumerary inserts were as follows: rHPIV3 1HN_{N-P} 2HN_{P-M} (Figure 11, sixth recombinant from the top) contained the HPIV1 HN and HPIV2 HN genes inserted between the N/P and P/M genes, respectively; rHPIV3 1HN_{N-P} 2HN_{P-M} HA_{HN-L} (Figure 11, seventh recombinant) contained the HPIV1 HN, HPIV2 HN, and measles virus HA inserted between the N/P, P/M, and HN/L genes, respectively; and rPIV3 1HN_{N-P} 2HN_{P-M} 3918GU_{HN-L} (Figure 11, bottom), contained the HPIV1 HN and HPIV2 HN genes inserted between the N/P and P/M genes, respectively, and in addition contained the 3918-nt GU insert between the HN and L genes.

It is noteworthy that the penultimate of these constructs, rHPIV3 1HN_{N-P} 2HN_{P-M} HA_{HN-L} (Figure 11, seventh construct from the top), contained protective antigens for four pathogens: HPIV3 (HN and F), HPIV1 (HN), HPIV2 (HN), and measles virus (HA). The total length of foreign sequence inserted into this recombinant was about 5.5 kb, which is 36% of the total HPIV3 genome length of 15,462 nt. The last recombinant, rHPIV3-1HN_{N-P}2HN_{P-M}GU_{HN-L} (Figure 11, bottom), was approximately 23 kb in length. This is 50% longer than wild-type HPIV3, and longer than any previously described biologically derived or recombinant paramyxovirus.

Recovery and replication *in vitro* of recombinant rHPIV3 bearing one, two, or three supernumerary gene inserts

The full length HPIV3 antigenomic cDNAs bearing single or multiple supernumerary genes of heterologous paramyxovirus protective antigens were separately transfected into HEp-2 monolayer cultures on six-well plates (Costar, Cambridge, MA) together with the support plasmids pTM(N), pTM(P no C), and pTM(L) and LipofectACE (Life Technologies, Gaithersburg, MD) and the cells were simultaneously infected with MVA-T7, a replication-defective vaccinia virus recombinant encoding the bacteriophage T7 polymerase protein using techniques previously described (Durbin et al., *Virology* 235:323-332, 1997a; Skiadopoulos et al., *Virology* 272:225-34, 2000, each incorporated herein by reference). After incubation at 32°C for up to four days, the transfection harvest was passaged onto LLC-MK2 monolayer cultures in a 25 cm² flask and the cells were incubated for 5 days at 32°C. The virus recovered from the cell supernatant was further passaged on LLC-MK2 cells at 32°C to amplify the virus. rHPIV3s bearing single or multiple foreign gene inserts were biologically-cloned by plaque purification on LLC-MK2 cells as previously described (Skiadopoulos et al., *J. Virol.* 73:1374-81, 1999a, incorporated herein by reference). Viral suspensions derived from biologically cloned virus were amplified on LLC-MK2 cells and yielded final titers of 10⁷ and 10⁹ TCID₅₀/ml, similar to the range of titers typically obtained for wt rHPIV3. Recombinant viruses were assayed for their ability to grow at 39°C. Surprisingly several rHPIV3s bearing single or multiple foreign gene insertions (rHPIV3 1HN_{N-P}, rHPIV3 1HN_{N-P}2HN_{P-M}HA_{HN-L}, and rHPIV3 1HN_{N-P} 2HN_{P-M} 3918 GU_{HN-L}) were 100 to 1000-fold restricted for replication at 39°C compared to the replication at the permissive temperature.

Viral RNA (vRNA) was isolated from biologically cloned recombinant chimeric viruses as described above (see also, Skiadopoulos et al., J. Virol. 73:1374-81, 1999a, incorporated herein by reference). This was used as the template for reverse transcription and polymerase chain reaction (RT-PCR) using specific primers that border the insertion sites. The amplified products were analyzed by restriction endonuclease digestion and partial DNA sequencing of the junction regions to confirm the presence and identity of each foreign insert. In all cases, the expected, correct sequence was confirmed.

Replication in the respiratory tract of hamsters of rHPIV3s expressing one, two, or three supernumerary foreign protective antigens

It was previously shown that rHPIV3 or rHPIV3-1 viruses expressing one supernumerary viral protective antigen gene replicated efficiently *in vitro* and *in vivo* and induced protective immune responses against both the vector virus and the virus represented by the supernumerary antigen gene. However, it was unknown whether a rHPIV could accommodate two or more supernumerary genes and retain the ability to replicate efficiently *in vitro* and *in vivo* and to induce protective immune responses against both the vector and the expressed supernumerary antigens. The present example indicates that this is indeed possible.

Hamsters in groups of eight were inoculated intranasally with 10^6 TCID₅₀ of each rHPIV3 bearing single or multiple supernumerary foreign gene inserts or with control viruses (Table 13). Nasal turbinates and lungs were harvested four days post infection and the virus present in tissue homogenates was quantified by serial dilution on LLC-MK2 monolayer cultures at 32°C as described above (see also, Skiadopoulos et al., J. Virol. 73:1374-81, 1999a). Virus was detected by hemadsorption with guinea pig erythrocytes, and the mean virus titer for each group is expressed as log₁₀ TCID₅₀(50% tissue culture infectious dose/gram tissue ± SE).

Table 13

Replication of recombinant HPIV3s containing single or multiple supernumerary gene inserts expressing the HPIV1, HPIV2 or measles virus glycoprotein genes in the upper and lower respiratory tract of hamsters

Group ^a no.	Virus ^b	Mean virus titer ^c (log ₁₀ TCID ₅₀ /g ± S.E.) in:			
		Nasal Turbinates	titer reduction (log ₁₀) ^d	Lungs	titer reduction (log ₁₀) ^d
1	rHPIV3 1HN _(N-P)	4.5 ± 0.2	1.8	3.9 ± 0.2	3.0
2	rHPIV3 1HN _(P-M)	3.5 ± 0.2	2.8	4.3 ± 0.2	2.3
3	rHPIV3 2HN _(N-P)	5.4 ± 0.2	0.9	5.3 ± 0.3	1.6
4	rHPIV3 2HN _(P-M)	6.3 ± 0.1	0.0	6.3 ± 0.5	0.6
5	rHPIV3 HA _(N-P)	5.3 ± 0.2	1.0	5.8 ± 0.4	1.1
6	rHPIV3 HA _(P-M)	6.0 ± 0.2	0.3	7.3 ± 0.2	-0.4
7	rHPIV3 HA _(HN-L)	6.0 ± 0.1	0.3	6.6 ± 0.2	0.3
8	rHPIV3 1HN _(N-P) 2HN _(P-M)	5.2 ± 0.1	1.1	5.0 ± 0.3	1.9
9	rHPIV3 1HN _(N-P) 2HN _(N-P) HA _(HN-L)	1.6 ± 0.1	4.7	2.5 ± 0.1	4.4
10	rHPIV3 1HN _(N-P) 2HN _(N-P) 3918 GU _(HN-L)	2.0 ± 0.3	4.3	1.8 ± 0.2	5.1
11	rHPIV3 <i>cp45</i>	4.6 ± 0.1	1.7	2.1 ± 0.2	4.8
12	rHPIV3 wt	6.3 ± 0.1	-	6.9 ± 0.1	-

a. 8 hamsters per group.

b. Each hamster was inoculated with 10⁶ TCID₅₀ of virus in a 0.1 ml inoculum.

c. Virus was titrated by serial dilution on LLC-MK2 monolayer cultures at 32 °C.

d. Reduction in virus replication compared to rHPIV3 wt (group 12).

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It was shown above that a rHPIV3 expressing measles virus HA from a supernumerary gene insert between the HPIV3 HN and L genes, between the N and P genes, or between the P and M genes was modestly (about 10 to 20-fold) restricted in replication in the upper and lower respiratory tract of hamsters. This was confirmed in the present experiment, in which rHPIV3 containing measles virus HA as a single supernumerary gene between the N/P, P/M or HN/L genes was attenuated up to 10-fold (Table 13, groups 5, 6, and 7). Similarly, insertion of the HPIV2 HN gene between the HPIV3 N and P genes or between the P and M genes also exhibited a modest reduction (about 10 to 20-fold) in replication in the respiratory tract of hamsters (Table 13 groups 3 and 4). In contrast, insertion of the HPIV1 HN gene between the P and M genes or between the N and P, resulted in over approximately 100-fold reduction of replication in the upper and lower respiratory tract of hamsters (Table 13, groups 1 and 2). Since the HPIV1 HN, HPIV2 HN, and measles virus HA gene insertions are all of approximately the same size (1794 nt, 1781 nt, and 1926 nt, respectively), this was unlikely to be due to insert length. Therefore, the greater level of attenuation conferred by the introduction the HPIV1 HN gene likely is due to an additional attenuating effect that is specific to the expression of the HPIV1 HN protein on replication of the HPIV3 vector. Thus, in some cases, such as with HPIV1 HN, a supernumerary antigen can attenuate rHPIV3 for hamsters above and beyond the modest attenuation due to inserting an additional gene.

Inspection of the data in Table 13 indicates that the site of insertion also plays a role in the level of restriction of replication of the chimeric rHPIV3 in the respiratory tract of hamsters. Insertion of the measles virus HA gene or the HPIV2 HN gene between the rHPIV3 N and P genes resulted in a greater reduction of replication in the upper and lower respiratory tract of hamsters than did insertion between the P and M genes (Table 13, compare groups 3 versus 4 and 5 versus 6). This site-specific attenuation effect on replication of the HPIV3 vector was not evident for insertions of the HPIV1 HN gene, presumably because it was masked by the more substantial attenuating effect specific to HPIV1 HN.

The rHPIV3 chimeric recombinant viruses exhibited a gradient of attenuation that was a function of the number of supernumerary gene inserts. The viruses bearing three added genes exhibited the greatest effect, and were reduced approximately 10,000 - 108,000 fold in replication in the upper and lower respiratory tract of the infected hamsters (Table 13, groups 9 and 10). The rHPIV3 chimeric recombinant virus bearing two gene inserts

exhibited an intermediate level of attenuation, and was reduced approximately 12-80 fold (Table 13, group 8). rHPIV3 chimeric recombinant viruses bearing one supernumerary gene (except those bearing the HPIV1 HN gene) were reduced only approximately 10-25 fold (groups 3-7 in Table 13). Importantly, rHPIV3 chimeric recombinant viruses bearing one, two, or three supernumerary gene inserts replicated in all animals. The most attenuated of these viruses, namely those bearing three supernumerary genes, were substantially more attenuated than rcp45 (group 11) with respect to replication in the upper and lower respiratory tract.

10 **Immunogenicity in hamsters of rHPIV3s expressing one, two, or three supernumerary foreign protective antigens**

Hamsters were infected with HPIV1 wt, HPIV2 wt, rHPIV3 wt, or rHPIV3s bearing single, double or triple supernumerary gene inserts as described above. Serum samples were collected 3 days pre-immunization and 28 days post-immunization and were assayed for HPIV1, HPIV2, HPIV3 or measles virus-specific antibodies by virus neutralizing assay specific for either HPIV1 or measles virus, or by the hemagglutination inhibition (HAI) assay for HPIV3 or HPIV2 HN-specific antibodies (Table 14). All rHPIV3 viruses elicited a strong immune response to the HPIV3 backbone with the exception of the viruses bearing the three supernumerary gene insertions. The reduced or absent immune response in hamsters infected with either the rHPIV3 1HN_{N-P} 2HN_{N-P} HA_{HN-L} or rHPIV3 1HN_{N-P} 2HN_{N-P} 3918GU_{HN-L} was likely a result of these viruses being overly attenuated for replication in hamsters. Likewise the immune response to the vectored antigens in the viruses bearing three foreign genes was also low or undetectable. In contrast, viruses bearing single or double foreign gene insertions induced an immune response against each of the additional antigens, demonstrating that the vectored foreign genes are immunogenic in hamsters, and as in the example of rHPIV3 1HN_{N-P} 2HN_{N-P} (Table 14; group 11) can be used to induce a strong immune response to three different viruses: HPIV1, HPIV2 and HPIV3.

Table 14

Immune response in hamsters to immunization with rHPiV3 vectors expressing single or multiple supernumerary protective antigens of HPiV1, HPiV2, or measles virus^a

Group no.	Virus	Serum ^b antibody titer (mean log ₂ ± SE) to the indicated virus			
		HPiV3 ^c	HPiV1 ^d	HPiV2 ^e	Measles virus ^f
1	rHPiV3 wt	10.0 ± 0	-	-	-
2	HPiV2 wt	<2.0 ± 0	-	8.0 ± 0.0	-
3	HPiV1 wt	<2.0 ± 0	5.4 ± 0.3	-	-
4	rHPiV3 HA _(N-P)	9.5 ± 0.2	-	-	12.4 ± 0.4
5	rHPiV3 HA _(P-M)	8.7 ± 1.4	-	-	11.8 ± 0.2
6	rHPiV3 HA _(HN-L)	9.0 ± 0	-	-	8.1 ± 0.6
7	rHPiV3 1HN _(N-P)	9.5 ± 0.2	3.4 ± 0.6	-	-
8	rHPiV3 1HN _(P-M)	7.2 ± 0.8	2.7 ± 0.3	-	-
9	rHPiV3 2HN _(N-P)	9.8 ± 0.5	-	9.3 ± 0.8	-
10	rHPiV3 2HN _(P-M)	10.0 ± 0.5	-	8.3 ± 1.1	-
11	rHPiV3 1HN _(N-P) 2HN _(N-P)	9.6 ± 0.7	5.5 ± 0.4	8.3 ± 0.8	-
12	rHPiV3 1HN _(N-P) 2HN _(N-P) HA _(HN-L)	<2.0 ± 0	1.0 ± 0.3	<2.0 ± 0.0	<3
13	rHPiV3 1HN _(N-P) 2HN _(N-P) 3918 GU _(HN-L)	4.3 ± 0.7	2.3 ± 0.6	<2.0 ± 0.0	-
14	rHPiV3 cp45	7.7 ± 0.2	-	-	-

a. Mean antibody response in groups of hamsters (n=6) inoculated intranasally with 10⁶ TCID₅₀ rHPiV3s expressing the hemagglutinin-neuraminidase protein of HPiV1 (1HN), HPiV2 (2HN) or measles virus hemagglutination (HA) inserted between the N and P genes (N-P), the P and M genes (P-M) or the HN and L genes (HN-L) of rHPiV3.

b. Sera were collected 3 days before and 28 days after immunization.

c. Mean hemagglutination inhibiting antibody (HAI) titer to HPiV3.

d. Mean neutralizing antibody titer to HPiV1.

e. Mean HAI antibody titer to HPiV2.

f. Mean neutralizing antibody titer to measles virus (60% plaque reduction neutralization, PRN).

EXAMPLE IX

Use of rHPIV3-N_B as an Attenuated Vector for the Measles Virus HA Protein.

The use of an animal virus that is attenuated in humans because of a host range restriction as a vaccine against an antigenically-related human counterpart is the basis of the Jennerian approach to vaccine development. The Kansas (Ka) strain of bovine parainfluenza virus type 3 (BPIV3) was found to be 100- to 1000-fold restricted in replication in rhesus monkeys compared to human parainfluenza virus type 3 (HPIV3), and was also shown to be attenuated in humans (Coelingh et al., J. Infect. Dis. 157:655-62, 1988; Karron et al., J. Infect. Dis. 171:1107-14, 1995b, each incorporated herein by reference). A viable chimeric recombinant human parainfluenza virus type 3 (HPIV3) virus was previously produced containing the nucleoprotein (N) open reading frame (ORF) from BPIV3 Ka in place of the HPIV3 N ORF. This chimeric recombinant was previously designated cKa-N (Bailly et al., J. Virol. 74:3188-3195, 2000a, incorporated herein by reference) and is referred to here as rHPIV3-N_B. This previous study was initiated with an exchange of the N ORF because, among the PIV3 proteins, the BPIV3 and HPIV3 N proteins possess an intermediate level of amino acid sequence identity (85%) (Bailly et al., Virus Genes 20:173-82, 2000b, incorporated herein by reference), and it was shown that such a BPIV3/HPIV3 N recombinant is viable (Bailly et al., J. Virol. 74:3188-3195, 2000a, incorporated herein by reference). This represents a "modified Jennerian" approach, in which only a subset of the genes in the vaccine virus is derived from the animal counterpart. rHPIV3-N_B grew to a titer comparable to that of the rHPIV3 and BPIV3 parent viruses in LLC-MK2 monkey kidney and Madin Darby bovine kidney cells (Bailly et al., J. Virol. 74:3188-3195, 2000a). Thus, the heterologous nature of the N protein did not impede replication of rHPIV3-N_B *in vitro*. However, rHPIV3-N_B was restricted in replication in rhesus monkeys to a similar extent as its BPIV3 parent virus (Bailly et al., J. Virol. 74:3188-3195, 2000a). This identified the BPIV3 N protein as a determinant of the host range restriction of replication of BPIV3 in primates.

The rHPIV3-N_B chimeric virus thus combines the antigenic determinants of HPIV3 with the host range restriction and attenuation phenotype of BPIV3. There are 79 differences out of a total of 515 amino acids between the N proteins of HPIV3 and BPIV3 (Bailly et al., Virus Genes 20:173-82, 2000b). Many of these 79 amino acid differences likely contribute to the host-range attenuation phenotype of rHPIV3-N_B. Because the host range restriction is anticipated to be based on numerous amino acid differences, it is

anticipated that the attenuation phenotype of rHPIV3-N_B will be stable genetically even following prolonged replication *in vivo*. Despite its restricted replication in rhesus monkeys, rHPIV3-N_B induced a high level of resistance to challenge of the monkeys with wild type (wt) HPIV3, and this level of resistance was indistinguishable from that conferred by immunization with wt rHPIV3. The infectivity, attenuation, and immunogenicity of rHPIV3-N_B suggest that this novel chimeric virus is an excellent candidate as a HPIV3 vaccine (Bailly et al., *J. Virol.* 74:3188-3195, 2000a). Furthermore, as described below, it is shown herein that such chimeric viruses are excellent candidates to serve as an attenuated vector for the expression of supernumerary protective antigens, such as the HA of measles virus. The vector component of the resulting chimeric virus induces an immune response against HPIV3, and the added supernumerary genes induce immune responses against their respective heterologous pathogens. In this specific example, a bivalent attenuated vaccine virus is made that simultaneously induces immune response to HPIV3 and measles virus.

It is shown above that rHPIV3 can be used as a vector for expression of the measles virus hemagglutinin (HA) protein. In two examples, *rcp45_L* HA(N-P) and *rcp45* HA(HN-L), attenuated vectors expressing the measles virus HA gene possessed three attenuating amino acid point mutations in the vector backbone. The rHPIV3-N_B vector of the present invention will likely be even more stable than vectors having an attenuation phenotype based on three amino acid point mutations. Also above, it was shown that the insertion of HA as a supernumerary gene into rHPIV3 conferred some attenuation on replication of both wt HPIV3 and attenuated HPIV3*cp45_L* for hamsters. In addition, the insertion of a 1908-nt gene insert into HPIV3 did not attenuate the wild type backbone, but did increase the level of attenuation of a backbone bearing the *cp45_L* mutations for replication in hamsters. Therefore, the insertion of the measles virus HA gene into the host-range restricted rHPIV3-N_B virus is projected to further attenuate its growth *in vitro* and/or *in vivo*. Inserts that affect replication *in vitro* or *in vivo* can be problematic for development of specific vaccines such as rHPIV3-N_B. Specifically, a candidate virus that is highly restricted in replication *in vitro* would be difficult to manufacture, and one that is highly restricted in replication *in vivo* could be overattenuated and not useful as a vaccine. It was also not known whether the rHPIV3-N_B chimeric virus expressing the measles virus HA glycoprotein, designated rHPIV3-N_B HA_(P-M), would be satisfactorily immunogenic in primates against both HPIV3 and measles virus since all previous studies with HPIV3 expressing HA were conducted in a rodent model.

The present example, which details the generation of rHPIV3-N_B HA_(P-M) using reverse genetic techniques, indicates, surprisingly, that insertion of the HA gene into rHPIV3-N_B did not further restrict its replication in rhesus monkeys. Presumably the attenuating effect of insertion is masked by the genetic elements present in the N_B gene that specify the host range restriction of replication in primates. Rather, rHPIV3-N_B HA_(P-M) was satisfactorily attenuated in rhesus monkeys. Immunization of rhesus monkeys with rHPIV3-N_B HA_(P-M) induced resistance to the replication of wt HPIV3 challenge virus and stimulated high levels of neutralizing antibodies to the measles virus, levels that are known to be protective in humans (Chen et al., *J. Infect. Dis.* 162:1036-42,1990, incorporated herein by reference).

Construction of a pFLC HPIV3-N_B HA_(P-M), a chimeric bovine/human PIV3 antigenomic cDNA encoding the BPIV3 N gene ORF in place of the rHPIV3 N gene ORF and the HA gene of measles virus as a supernumerary gene inserted between the rHPIV3 P and M genes

The full length antigenomic cDNA plasmid pFLC HPIV3-N_B HA_(P-M) (Figure 12) was constructed in two steps. First, the previously-constructed pLeft-N_B plasmid contains the 3' half of the HPIV3 antigenomic cDNA (HPIV3 nts 1-7437, the latter position being an XhoI site within the HN gene) with the HPIV3 N ORF replaced by that of BPIV3 (Bailly et al., *J. Virol.* 74:3188-3195, 2000a, incorporated herein by reference). The *PshAI*-*NgoMIV* fragment was excised from this plasmid. Note that the *PshAI* site is at position 2147 in the HPIV3 antigenome sequence (see Figure 12) and the *NgoMIV* site occurs in the vector sequence, and so this removes all of the HPIV3 sequence downstream of the *PshAI* site. This fragment was replaced by the *PshAI*-*NgoMIV* fragment from the previously-constructed plasmid pLeft HA_(P-M), which contains the measles virus HA ORF under the control of HPIV3 transcription signals and inserted between the HPIV3 N and P genes (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference). This yielded pLeft-N_B HA_(P-M). Next, the 11899 nt *NgoMIV* to *Xho* I fragment of pLeft N_B HA_(P-M), containing the 3' half of the HPIV3 antigenomic cDNA including the BPIV3 N gene ORF and the measles virus HA gene insert, was cloned into the *NgoMIV* to *Xho* I window of pRight, a plasmid encoding the 5' half of the HPIV3 antigenomic cDNA (PIV3 nts 7462-15462) (Durbin et al., *Virology* 235:323-332, 1997a). This yielded pFLC HPIV3-N_B HA_(P-M), a plasmid bearing the full length antigenomic cDNA of HPIV3 containing the BPIV3 N ORF

in place of the HPIV3 N ORF, and containing measles virus HA gene as a supernumerary gene inserted between the P and M genes of HPIV3.

Recovery of chimeric rHPIV3 expressing the bovine N gene and the measles virus HA gene

rHPIV3-N_B HA_{P-M} was recovered from HEp-2 cells transfected with pFLC HPIV3-N_B HA_{P-M}. To accomplish this, pFLC HPIV3-N_B HA_{P-M} was transfected into HEp-2 cells on six-well plates (Costar, Cambridge, MA) together with the support plasmids pTM(N), pTM(P no C), and pTM(L) and LipofectACE (Life Technologies, Gaithersburg, MD), and the cells were simultaneously infected with MVA-T7, a replication-defective vaccinia virus recombinant encoding the bacteriophage T7 polymerase protein, as described above. After incubation at 32°C for four days, the transfection harvest was passaged onto LLC-MK2 cells in a 25 cm² flask, and the cells were incubated for 5 days at 32°C. The virus recovered from the cell supernatant was amplified by a further passage on LLC-MK2 cells at 32°C. rHPIV3-N_B HA_{P-M} was biologically cloned by plaque purification on LLC-MK2 monolayer cultures as described above. Viral suspensions derived from biologically cloned virus were amplified on LLC-MK2 monolayer cultures at 32°C. Viral RNA (vRNA) was isolated from biologically cloned recombinant chimeric viruses as described above. RT-PCR was performed using specific oligonucleotide primer pairs spanning the BPIV3 N ORF or the measles virus HA gene, and the amplified cDNAs were analyzed by restriction endonuclease digestion and partial DNA sequencing as described above. This confirmed the presence of the BPIV3 N ORF substitution and the measles virus HA supernumerary gene insert.

Expression of the measles virus HA protein was initially confirmed by immunostaining plaques formed on LLC-MK2 monolayer cultures infected with rHPIV3-N_B HA_{P-M} using mouse monoclonal antibodies specific to the measles virus HA protein and goat anti-mouse peroxidase-conjugated antibodies, as described previously (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference).

rHPIV3-N_B HA_{P-M} replicates to the same level as rHPIV3-N_B in the respiratory tract of rhesus monkeys.

It was next determined whether the acquisition of the measles virus HA insert significantly decreased the replication of rHPIV3-N_B in the upper and lower respiratory tract, as was observed when a supernumerary gene was inserted into an attenuated HPIV3 backbone lacking a bovine chimeric component. It was also determined whether rHPIV3-N_B HA_{P-M} replicated sufficiently to induce an immune response against both HPIV3 and measles virus *in vivo*. The replication of rHPIV3-N_B HA_{P-M} in the upper and lower respiratory tract of rhesus monkeys was compared to that of its rHPIV3-N_B parent as well as wt HPIV3 and wt BPIV3 (Table 15). Rhesus monkeys that were seronegative for both HPIV3 and measles virus were inoculated simultaneously by the intranasal (IN) and intratracheal (IT) routes with one milliliter per site of L15 medium containing 10⁵ TCID₅₀ of virus suspension, as described previously (Bailly et al., *J. Virol.* **74**:3188-3195, 2000a). Nasopharyngeal (NP) swab samples were collected on days 1 through 10 post-infection, and tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10 post-infection. Virus present in the NP and TL specimens was quantified by serial dilution on LLC-MK2 cell monolayers at 32°C, and the titer obtained was expressed as log¹⁰ TCID₅₀/ml (Table 15).

This comparison showed that the rHPIV3-N_B HA_(P-M) chimeric virus replicated to the same level in the upper and lower respiratory tract of rhesus monkeys as its rHPIV3-N_B parent virus. This level of replication was also comparable to that of the BPIV3 virus parent, demonstrating that rHPIV3-N_B HA_(P-M) retains the attenuation phenotype of rHPIV3-N_B and BPIV3 and, unexpectedly, that the insertion of the measles virus HA gene into the rHPIV3-N_B genome does not further attenuate this virus for replication in the respiratory tract of rhesus monkeys.

Table 15

A chimeric human/bovine PIV3 containing the measles virus hemagglutinin gene is satisfactorily attenuated for replication in the upper and lower respiratory tract of rhesus monkeys, induces antibodies to both HPIV3 and measles virus, and protects against HPIV3 wild type virus challenge

Group no.	Immunizing virus ^a	No. of animals ^a	Response to immunization			Response to challenge with HPIV3 wt on day 28 or 31		Response to administration of measles virus vaccine (Moraten) on day 59
			Virus Replication		Serum antibody response	Virus replication	Serum antibody response	
			Mean peak virus titer ^c (log ₂ ±TCID ₅₀ /ml ± SE)	Tracheal lavage	Serum HAI antibody titer (mean ± S.E.) for HPIV3 on day 28/31 ^{d,e}	Mean peak HPIV3 virus titer ^e (log ₁₀ TCID ₅₀ /ml ± SE)	HAI antibody titer (mean reciprocal log ₂ ± SE) for HPIV3 on day 56/59 ^h	
1	rHPIV3 wt	6	4.9 ± 0.4	3.2 ± 0.6	9.3 ± 0.6	0.5 ± 0.0	0.5 ± 0.0	8.2 ± 0.8
2	rHPIV3-N _B	8	2.6 ± 0.6	2.0 ± 0.4	7.3 ± 0.3	1.4 ± 0.9	0.5 ± 0.0	10.1 ± 0.4
3	rHPIV3-N _B HA _(P-M)	4	2.2 ± 0.6	2.8 ± 0.6	6.8 ± 0.3	1.2 ± 0.7	2.3 ± 0.2	10.2 ± 0.4
4	BPIV3 Ka	8	2.3 ± 0.2	1.9 ± 0.2	5.0 ± 0.4	2.9 ± 0.3	2.0 ± 0.5	ND
5	none ^b	4	ND	ND	< 2	4.5 ± 0.3	4.5 ± 0.2	ND

a. The present study included 4 monkeys that received rHPIV3-N_B HA_(P-M) and two monkeys in each of the groups that received rHPIV3 wt, rHPIV3-N_B, or BPIV3 Ka. With the exception of the group that received rHPIV3-N_B HA_(P-M), the data presented includes historical data from studies reported in Bailey et al., *J. Virol.* 74:3188-3195, 2000, and Schmidt et al., *J. Virol.* 74:8922-8929, 2000.

b. Historical data from Schmidt et al., *J. Virol.* 74:8922-8929, 2000.

c. Monkeys were inoculated intranasally and intratracheally with 10⁵ TCID₅₀ of virus in a 1 ml inoculum at each site. Nasopharyngeal (NP) swab samples were collected on days 1 to 10 post-infection. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10 post-infection. Mean of the peak virus titers for each animal in its group irrespective of sampling day. S.E. = standard error. Virus titrations were performed on LLC-MK2 cells at 32°C. The limit of detection of virus titer was 10 TCID₅₀/ml.

d. In the present study sera were collected from monkeys on day 31 post immunization and animals were then challenged with HPIV3. In the two previous studies, monkeys were sampled and challenged on day 28 post immunization.

e. Sera collected for the present study and from the two previous studies were assayed at the same time. Serum HAI titer is expressed as the mean reciprocal log₂ ± standard error, SE.

- f. Animals were immunized on day 59 with 10^6 pfu of the measles virus Moraten vaccine strain administered parenterally (IM). Serum was collected 28 days later (i.e., 87 days after the first immunization). Data shown was obtained from samples collected only from animals in the present study. Mean neutralizing antibody titer to wt measles virus is expressed as the mean reciprocal \log_2 standard error. PRN, plaque reduction neutralizing.
- g. 28 or 31 days after immunization monkeys were inoculated intranasally and intratracheally with 10^6 TCID₅₀ of wt HPIV3 in a 1 ml inoculum at each site. NP and TL samples were collected on days 0, 2, 4, 6 and 8 post challenge. The titers obtained for NP and TL samples on day 0 were $< 2.0 \log_{10}$ TCID₅₀/ml.
- h. With the exception of group 5, data shown are from the present study.

Immunization of rhesus monkeys with rHPIV3-N_B HA_{P-M} induces high titers of antibodies against both HPIV3 and measles virus and protects the monkeys from challenge with HPIV3

5 Rhesus monkeys immunized with rHPIV3-N_B HA_{P-M} developed high levels of serum antibodies against both HPIV3 and measles virus (Table 15). Serum HPIV3 antibodies were quantified by hemagglutination inhibition assay (HAI) using guinea pig erythrocytes as described previously (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference), and the titers are expressed as mean reciprocal log₂ ± SE. High levels
10 of serum HAI antibodies to HPIV3 were induced by both rHPIV3-N_B HA_{P-M} and rHPIV3-N_B, demonstrating that these attenuated recombinants can induce a strong immune response against the backbone antigens of the HPIV3 vector. It was also found that rhesus monkeys immunized with rHPIV3-N_B HA_{P-M} developed high levels of serum measles virus neutralizing antibodies 31 days after immunization, levels that are in excess of those needed
15 to protect humans against infection with measles virus (Chen et al., *J. Infect. Dis.* 162:1036-42, 1990, incorporated herein by reference). Serum neutralizing antibody titers against wild type measles virus were quantified as described previously (Durbin, *J. Virol.* 74:6821-31, 2000), and the titers are expressed as reciprocal mean log₂ ± SE (Table 15).

To compare the ability of infection with the live attenuated rHPIV3-N_B HA_{P-M} and rHPIV3-N_B virus vaccine candidates to protect against wt HPIV3, the monkeys were
20 challenged IN and IT with 10⁶ TCID₅₀ of wt HPIV3 31 days after the initial infection (Table 15). Nasopharyngeal swab and tracheal lavage samples were collected on days 2, 4, 6, and 8 post-challenge. Virus present in the specimens was quantified by serial dilution on LLC-MK2 monolayer cultures as described above. rHPIV3-N_B HA_{P-M} and rHPIV3-N_B conferred
25 a comparable, high level of protection against challenge with wt HPIV3 as indicated by a 100 to 1000-fold reduction in wt HPIV3 replication in the respiratory tract of immunized monkeys. This demonstrated that insertion of the measles virus HA gene into the chimeric bovine/human PIV3 did not diminish the level of protection induced by the HPIV3 glycoproteins present in the backbone of the attenuated virus vector.

30 Immunogenicity of rHPIV3-N_B HA_{P-M} was then compared with that of the licensed Moraten strain of live attenuated measles virus vaccine in rhesus monkeys, a species in which both PIV3 and measles virus replicate efficiently. Rhesus monkeys previously infected with a rHPIV3 virus or with rHPIV3-N_B HA_{P-M} were immunized parenterally (IM)

with 10^6 pfu of the Moraten strain of live-attenuated measles virus vaccine on day 59, and serum samples were taken on day 87 and analyzed for neutralizing antibodies against measles virus (Table 15). In animals that were naïve for measles virus before receiving the Moraten vaccine (Table 15, groups 1 and 2), the titer of measles-specific antibodies induced by the Moraten vaccine was approximately the same as that observed in rHPIV3-N_B HA_{P-M}-immunized animals (Table 15, group 2). Thus, rHPIV3-N_B HA_{P-M} vector expressing the HA glycoprotein measles virus was equivalent to the Moraten vaccine in the ability to induce virus-neutralizing antibodies in this primate model.

An important advantage of rHPIV3-N_B HA_{P-M} as a vaccine for measles virus over the Moraten vaccine is that the PIV vector can be administered by the intranasal route, whereas live-attenuated measles virus vaccines are not consistently infectious by this route, probably as a consequence of their attenuation and adaptation to cell culture. This makes it possible to immunize with rHPIV3-N_B HA_{P-M} in early infancy, an age group that cannot be immunized with a current live attenuated measles virus vaccine such as the Moraten strain because of the neutralizing and immunosuppressive effects of maternal antibodies (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference). Other advantages are also described above, including the superior growth of the PIV vector in cell culture and the lack of incorporation of measles virus HA in the virions, which should preclude changing the tropism of the PIV vector and should preclude measles virus-induced immunosuppression.

The lack of effective vaccination against measles virus infection results in the deaths of over 2700 children every day worldwide. The rHPIV3-N_B HA_(P-M) candidate vaccine offers a unique opportunity to immunize against two major causes of severe pediatric disease, namely, HPIV3 and measles virus. Unlike the currently licensed measles virus vaccines, we expect that chimeric rHPIV3-N_B HA_(P-M) and other human-bovine chimeric vector constructs, expressing the major antigenic determinant of measles virus or other heterologous pathogens, can be used to induce a strong immune response to, e.g., measles virus, in infants and children younger than six months of age (Durbin, *J. Virol.* 74:6821-31, 2000). An effective immunization strategy for infants and children will be required to meet the World Health Organization goal to eradicate measles by the year 2010. In particular, it would be advantageous for eradication to use a measles virus vaccine that does not involve infectious measles virus.

EXAMPLE X

Use of a Recombinant Bovine-Human Parainfluenza Virus Type 3 (rB/H PIV3) as a Vector for RSV Glycoprotein Supernumerary Genes

For use within the present invention, a recombinant chimeric human-bovine
5 PIV was constructed in which the BPIV3 F and HN genes were replaced with those of
HPIV3. This recombinant chimeric bovine-human virus rB/HPIV3 was shown to be fully
competent for replication in cell culture, whereas in rhesus monkeys it displayed the host
range-restricted, attenuated phenotype characteristic of BPIV3 and was highly immunogenic
and protective (U.S. Patent Application Serial No. 09/586,479, filed June 1, 2000 by
10 Schmidt et al.; Schmidt et al., *J. Virol.* 74:8922-9, 2000, each incorporated herein by
reference). This is another example of a "modified Jennerian" approach that is useful within
the compositions and methods of the invention, but in this case the entire set of viral
"internal" genes is derived from BPIV3, with the antigenic determinants alone derived from
HPIV3.

15 As noted above, there are numerous practical and safety considerations that
favor vaccines based on a single PIV3 backbone, as opposed to a complex mixture of
different viruses each of which must be separately attenuated and verified and which can
interact in unpredictable ways. In addition, the host range restriction of BPIV3 confers an
attenuation phenotype that should be very highly stable. In the present example, a
20 recombinant chimeric human-bovine PIV3 (rB/HPIV3) was designed, rescued and
characterized that encodes the respiratory syncytial virus (RSV) G or F glycoprotein, which
are the major RSV neutralization and protective antigens. This example shows that
rB/HPIV3 readily accepted the foreign RSV genes without a significant reduction of its
replicative efficiency *in vitro* or *in vivo* and thus is a promising candidate vaccine and vector.
25 This vector will be free of the problems of poor growth *in vitro* and instability that are
otherwise characteristic of RSV.

Construction of antigenomic cDNAs encoding recombinant chimeric rB/HPIV3 viruses bearing an RSV subgroup A G or F ORF as an additional, supernumerary gene

30 A full length cDNA of the BPIV3 Kansas strain was constructed in which the
F and HN glycoprotein genes of the bovine virus had been replaced with the corresponding
genes of the HPIV3 JS strain (rB/HPIV3) (U.S. Patent Application Serial No. 09/586,479,

filed June 1, 2000 by Schmidt et al.; Schmidt et al., J. Virol. 74:8922-9, 2000, each incorporated herein by reference). For use within the present invention, this cDNA was modified to contain three additional unique restriction enzyme recognition sites. Specifically, a *BlnI* site was introduced preceding the N ORF (nucleotide (nt) 103-109), an *AscI* site was introduced preceding the N gene end sequence and a *NotI* site was introduced preceding the P gene end sequence. These restriction enzyme recognition sites were introduced to facilitate the insertion of foreign, supernumerary genes into the genome of the chimeric B/HPIV3 virus genome. The sites were designed so that they did not disrupt any of the BPIV3 replication and transcription cis-acting elements. This specific example will describe insertion into the *BlnI* site (Figure 13).

The previously described RSV subgroup A glycoprotein genes G and F (GenBank accession no. M74568) were modified for insertion into the promoter-proximal *BlnI* site of B/HPIV3 (Figure 13). The strategy was to express each heterologous ORF as an additional, separate mRNA, and hence it was important that it be introduced into the rB/HPIV3 genome so that it was preceded by a BPIV3 gene start signal and followed by a BPIV3 gene end signal. The *BlnI* insertion site followed the gene start signal of the N gene (Figure 13). Hence, for insertion at this site, the RSV ORF needed to be modified by insertion of a *BlnI* site at its upstream end and addition of a BPIV3 gene end signal, intergenic region, gene start signal, and *BlnI* site at its downstream end. For the RSV A G ORF, the forward PCR primer used was (5' to 3')

AATTCGCTTAGCGATGTCCAAAAACAAGGACCAACGCACCGC (SEQ ID NO. 30),
the reverse primer was (5' to 3')

AAAAAGCTAAGCGCTAGCCTTTAATCCTAAGTTTTCTTACTTTTTTACTACTG
GC GTGGTGTGTTGGGTGGAGATGAAGGTTGTGATGGG (SEQ ID NO. 31)(*BlnI* site underlined, ORF translational initiation and termination triplets in bold). For the RSV A F ORF, the forward PCR primer used was (5' to 3')

AAAGGCCTGCTTAGCAAAAAGCTAGCACAATGGAGTTGCTAATCC
TCAAAGCAAAT GCAATTACC (SEQ ID NO. 32), and the reverse primer was (5' to 3')

AAAAGCTAAGCGCTAGCTTCTTTAATCCTAAGTTTTCTTACTTTTATTAGTTACT
AAATGCAATATTATTTATACCACTCAGTTGATC (SEQ ID NO. 33)(*BlnI* site underlined, ORF translational initiation and termination triplets in bold).

The PCR products were digested with *BlnI* and cloned into the modified full length cDNA clone using standard molecular cloning techniques. The resulting full length cDNA containing the RSV A G ORF was designated pB/HPIV3-G_A1 and the plasmid

containing the F ORF was designated pB/HPIV3-F_A1. The nucleotide sequence of each inserted gene was confirmed by restriction enzyme digestion and automated sequencing. All constructs were designed so that the final genome nucleotide length was a multiple of six, which has been shown to be a requirement for efficient RNA replication (Calain et al., *J. Virol.* 67:4822-30, 1993, incorporated herein by reference).

Recovery of rB/HPIV3-G1 and rB/HPIV3-F1 chimeric viruses from cDNA.

rB/HPIV3-G1 and rB/HPIV3-F1 viruses were recovered from the cDNAs pB/HPIV3-G_A1 and pB/HPIV3-F_A1, respectively. This was accomplished by the previously-described method in which HEp-2 cells were transfected with the respective antigenomic cDNA together with BPIV3 N, P and L support plasmids. The cells were simultaneously infected with a recombinant vaccinia virus, strain MVA, expressing the T7 RNA polymerase gene. The recovered recombinant viruses were cloned biologically by sequential terminal dilution in Vero cells. The presence of the inserted RSV G or F gene in the backbone of each recovered recombinant virus was confirmed by RT-PCR of viral RNA isolated from infected cells followed by restriction enzyme digestion and DNA sequencing. The sequence of the inserted gene and flanking regions in the recovered recombinant viruses was identical to that of the starting antigenomic cDNA.

rB/HPIV3-G1 and rB/HPIV3-F1 viruses replicate efficiently in cell culture.

The multicycle growth kinetics of rB/HPIV3-G1 and rB/HPIV3-F1 in LLC-MK2 cells were determined by infecting LLC-MK2 cell monolayers in triplicate at a multiplicity of infection (MOI) of 0.01 and harvesting samples at 24-hour intervals over a seven day period, as previously described (Baillly et al., *J. Virol.* 74:3188-3195, 2000a, incorporated herein by reference). These two viruses were compared with BPIV3 Ka, HPIV3 JS, rBIV3 Ka, and rB/HPIV3 (Figure 14). The two parental viruses bearing HPIV3 glycoproteins, namely HPIV3 and rB/HPIV3, appeared to replicate somewhat more rapidly than the others. However, the final titer achieved for each of the six viruses were similar with one exception: rB/HPIV3-F1 was approximately 8-fold reduced in its replicative capacity compared to the other viruses (Figure 14). This might be an effect of having this large gene in a promoter-proximal position, or might be an effect of the expression of a second fusogenic protein, or both. This latter possibility was suggested by the observation that rB/HPIV3-F1 induced massive syncytia, comparable to what is observed with wild type

RSV infection and greater than that observed with rB/HPIV3 or the other parental viruses. In comparison, rB/HPIV3-G1 induced less cytopathic effect and few syncytia in LLC-MK2 cells, comparable to rB/HPIV3. Nonetheless, rB/HPIV3-F1 and rB/HPIV3-G1 grew to a final titer of at least 10^7 TCID₅₀/ml in LLC-MK2 cells and in Vero cells. This indicates that each virus is fully-permissive for growth which will allow cost-efficient vaccine manufacture.

The rB/HPIV3-G1 and rB/HPIV3-F1 viruses replicate efficiently in the respiratory tract of hamsters

rB/HPIV3-G1 and rB/HPIV3-F1 were evaluated for their ability to replicate in the upper and lower respiratory tract of hamsters. The rB/HPIV3 parental virus, as well as the BPIV3 and HPIV3 biologically-derived viruses, were compared in parallel as controls (Table 16). Each virus was administered intranasally at a dose of 10^6 TCID₅₀, and one group received both rB/HPIV3-G1 and rB/HPIV3-F1. Animals from each group were sacrificed on days 4 and 5 post infection, and the virus titer in the nasal turbinates and lungs were determined by serial dilution. The level of replication of rB/HPIV3-G1 in the respiratory tract was very similar to, and statistically indistinguishable from, that of HPIV3 JS and BPIV3 Ka. Replication of rB/HPIV3-F1 appeared to be somewhat reduced on days 4 and 5 relative to the others, but this difference was not statistically significant in comparison with the biological BPIV3 virus, which in previous primate and clinical studies replicated sufficiently well to induce a protective immune response (Coelingh et al., *Virology* 162:137-143, 1988; Karron et al., *Pediatr. Infect. Dis. J.* 15:650-654, 1996, each incorporated herein by reference). Also, the titer of virus from the mixed infection of rB/HPIV3-G1 and rB/HPIV3-F1 appeared to be somewhat reduced in the lower respiratory tract on day 4, but this was not statistically significant. Replication of one of the control viruses, BPIV3 Ka, was somewhat reduced in the lower respiratory tract on day 5: this also was not statistically significant, and indicates that these small differences likely are not important. Thus, the rB/HPIV3-G1 and rB/HPIV3-F1 viruses appeared to be fully competent for replication *in vivo*, despite the presence of the 0.9 kb G or 1.8 kb F supernumerary gene next to the promoter.

Table 16

rB/HPIV3 bearing the RSV G or F ORF as a supernumerary gene in the promoter proximal position replicates efficiently in the respiratory tract of hamsters.

Immunizing virus ^a	Number of animals	Mean virus titer on day 4 ^b (log ₁₀ TCID ₅₀ /g ± S.E.) ^c		Mean virus titer on day 5 ^b (log ₁₀ TCID ₅₀ /g ± S.E.) ^c	
		Nasal turbinates	Lungs	Nasal turbinates	Lungs
rB/HPIV3-G1	6	5.9±0.1 (AB)	5.1±0.6 (A)	5.5±0.2 (A)	5.6±0.4 (AC)
rB/HPIV3-F1	6	5.1±0.3 (B)	4.6±0.2 (A)	5.7±0.2 (AB)	3.6±0.2 (BD)
rB/HPIV3-G1 & rB/HPIV3-F1	6	5.7±0.3 (BC)	4.3±0.8 (A)	5.6±0.2 (A)	5.9±0.2 (A)
rB/H PIV3	6	6.2±0.2 (AC)	5.2±0.6 (A)	6.5±0.1 (B)	5.7±0.6 (AC)
HPIV3 JS wild type	6	6.6±0.1 (A)	6.5±0.1 (A)	6.0±0.2 (AB)	6.0±0.4 (A)
BPIV3 Ka wild type	6	5.8±0.1 (AB)	6.1±0.2 (A)	5.3±0.2 (A)	4.2±0.5 (CD)

^a Hamsters were inoculated intranasally with 10⁶ TCID₅₀ of virus in a 0.1 ml inoculum.

^b Animals were sacrificed on day 4 or 5 post inoculation, as indicated, and virus titers in the nasal turbinates and lungs were determined by titration on LLC-MK2 (PIV3) or HEP-2 (RSV) cells at 32°C. The limit of detectability of virus was 10^{2.45} TCID₅₀/g tissue. S.E. = standard error.

^c Mean virus titers were assigned to similar groups (A, B, C, D) by the Tukey-Kramer test. Within each column, mean titers with different letters are statistically different (p<0.05). Titters indicated with two letters are not significantly different from those indicated with either letter.

The rB/HPIV3-G1 and rB/HPIV3-F1 viruses induce serum antibodies to both HPIV3 and RSV

Hamsters were infected with rB/HPIV3-G1, rB/HPIV3-F1, or rB/HPIV3 as described above. An additional group received both rB/HPIV3-G1 and rB/HPIV3-F1, and another group was infected intranasally with RSV. Serum samples were collected 5 days post infection and assayed for RSV-specific antibodies by an ELISA test specific for the RSV F protein or RSV G protein (Table 17), and for HPIV3 HN-specific antibodies by the hemagglutination inhibiting (HAI) antibody assay (Table 18). The titer of F-specific or G-specific antibodies induced by the rB/HPIV3-F1 or rB/HPIV3-G1 virus, respectively, was 2- to 4-fold higher than that induced by wild type RSV. Animals inoculated with both rB/HPIV3-F1 and rB/HPIV3-G1 also had high titers of F-specific and G-specific antibodies. In addition to high ELISA titers against RSV G and F, rB/HPIV3-G1 and rB/HPIV3-F1 also induced RSV-neutralizing serum antibody titers that were higher than those induced by wt RSV (Table 18). Each of the viruses induced a titer of PIV3-specific antibody that was indistinguishable from that of their parent virus rB/HPIV3 (Table 18). Thus, the rB/HPIV3 vector bearing the F or G gene of RSV induced strong immune responses against both the RSV insert and the PIV vector.

Table 17

Immunization of hamsters with rB/HPIV3 expressing the RSV G or F ORF as a supernumerary gene induces an antibody response against the RSV G or F protein.

Immunizing virus ^a	Animals per group	Serum IgG ELISA titer against		Serum IgG ELISA titer against RSV F protein ^b		log ₂ -fold increase
		RSV G protein ^b		(mean recip. log ₂ ±S.E.)		
		Pre	Day 26	Pre	Day 26	
rB/HPIV3-G1	12	6.0 ± 0.4 ^c	12.5±0.5	6.7 ± 0.5 ^c	7.5±0.5	0.8
rB/HPIV3-F1	12	6.3 ± 0.3	7.2±0.3	6.8 ± 0.3	16.2±0.5	9.4
rB/HPIV3-G1 & rB/HPIV3-F1	12	6.5 ± 0.6	12.0±0.9	7.3 ± 0.5	14.7±0.4	7.4
rB/HPIV3	12	6.5 ± 0.4	8.0±0.4	7.3 ± 0.7	8.3±0.8	1.0
RSV	12	6.8 ± 0.3	10.8±0.4	7.3 ± 0.5	15.7±0.4	8.2

^a Hamsters were inoculated intranasally with 10⁶ TCID₅₀ of the virus in a 0.1 ml inoculum.

^b Serum samples were taken on day 26 post inoculation and analyzed by glycoprotein-specific ELISA for antibodies against FSV G or F protein, as indicated.

^cTiters in the pre serum specimen represent non-specific background levels of antibody in this sensitive ELISA.

Table 18

Immunization of hamsters with rB/HPIV3s expressing the RSV G or F ORF induces neutralizing antibodies against RSV as well as hemagglutination-inhibiting (HAI) antibodies against HPIV3.

Immunizing virus ^a	Animals per group	Serum neutralizing antibody response to RSV ^b		Serum HAI antibody response to HPIV3 ^c	
		(mean recip. log ₂ ±S.E.) ^d		(mean recip. log ₂ ±S.E.) ^d	
		Pre	Day 26	Pre	Day 26
rB/HPIV3-G1	12	≤ 3.3	10.0±0.3 (A)	≤ 2	10.0±0.5 (A)
rB/HPIV3-F1	12	≤ 3.3	9.3±0.5 (A)	≤ 2	8.8±0.1 (A)
rB/HPIV3-G1 & rB/HPIV3-F1	12	≤ 3.3	10.8±0.4 (A)	≤ 2	8.8±0.3 (A)
rB/HPIV3	12	≤ 3.3	0.8±0.8 (B)	≤ 2	9.5±0.8 (A)
RSV	12	≤ 3.3	8.1±1.2 (A)	≤ 2	≤ 2 (B)

^a Hamsters were inoculated intranasally with 10⁶ TCID₅₀ of the indicated PIV3 or 10⁶ PFU of RSV in a 0.1 ml inoculum.

^b Serum samples were taken on day 26 post inoculation and antibody titers were determined by 60% plaque reduction neutralization test.

^c Serum samples were taken on day 26 post inoculation and antibody titers were determined by hemagglutination inhibition test.

^d Mean virus titers were assigned to similar groups (A, B) by the Tukey-Kramer test. Within each column, mean titers with different letters are statistically different (p<0.05).

The rB/HPIV3-G1 and rB/HPIV3-F1 viruses induce resistance to replication of HPIV3 and RSV challenge virus.

Hamsters immunized with rB/HPIV3, rB/HPIV3-G1, rB/HPIV3-F1, or
5 rB/HPIV3-G1 plus rB/HPIV3-F1 vaccine candidates were challenged 28 days later by the
intranasal inoculation of 10^6 TCID₅₀ of HPIV3 or 10^6 PFU of RSV. The animals were
sacrificed five days later and the nasal turbinates and lungs were harvested and virus titers
determined (Table 19). Animals that had received the parental rB/HPIV3 virus or the G1
and F1 derivatives exhibited a high level of resistance to the replication of the HPIV3
10 challenge virus, and there were no significant differences between experimental groups.
Animals that received rB/HPIV3-G1, or rB/HPIV3-F1, or both viruses, exhibited a high
level of resistance to replication of the RSV challenge virus. The level of protective efficacy
of the rB/HPIV3-F1 virus against the RSV challenge appeared to be marginally less than that
of the rB/HPIV3-G1 virus or of the RSV control. However, this difference was not
15 significantly different. Thus, the rB/HPIV3 vector bearing either the F or G gene of RSV
induced a level of protective efficacy that was comparable to that of complete infectious
RSV.

Table 19

Immunization of hamsters with rB/HPIV3-G1 and or rB/HPIV3-F1 induces resistance to challenge with HPIV3 and RSV 28 days post infection.

Immunizing virus ^a	No. of Animals	Mean HPIV3 titer ^b (log ₁₀ TCID ₅₀ /g ± S.E.) ^d		Mean RSV titer ^c (log ₁₀ PFU/g ± S.E.) ^d	
		Nasal turb.	Lungs	Nasal turb.	Lungs
rB/HPIV3-G1	6	2.3±0.1 (A)	3.1±0.2 (A)	1.9±0.2 (AB)	≤ 1.7 (A)
rB/HPIV3-F1	6	2.6±0.2 (A)	3.1±0.1 (A)	2.9±0.4 (BC)	2.1±0.2 (A)
rB/HPIV3-G1 & rB/HPIV3-F1	6	2.8±0.2 (A)	2.8±0.3 (A)	1.8±0.1 (A)	1.9±0.4 (A)
rB/HPIV3	6	2.3±0.5 (A)	3.6±0.4 (A)	4.1±0.5 (C)	3.5±0.4 (B)
RSV	6	5.6±0.2 (B)	5.2±0.2 (B)	1.9±0.3 (AB)	≤ 1.7 (A)

^a Groups of 6 hamsters were inoculated intranasally with 10⁶ TCID₅₀ of the indicated PIV3 or 10⁶ PFU of RSV in a 0.1 ml inoculum.

^b HPIV3 titrations were performed on LLC-MK2 cells. The limit of detectability of virus was 10^{1.7} TCID₅₀/g tissue.

^c Quantitation of RSV was determined by plaque numeration on HEp-2 cells. The limit of detectability of virus was 10^{1.7} PFU/g tissue.

^d Mean virus titers were assigned to similar groups (A, B, C) by the Tukey-Kramer test. Within each column, mean titers with different letters are statistically different (p<0.05). Titers indicated with two letters are not significantly different from those indicated with either letter.

EXAMPLE XI

Use of rB/HPIV3.1 as a Vector for the Hemagglutinin HN and F Proteins of PIV2.

The chimeric rHPIV3-1 virus, which has a HPIV3 backbone in which the
5 HPIV3 HN and F genes have been replaced by their HPIV1 counterparts, serves as a useful
vector for the HPIV2 HN protein as a supernumerary gene. This chimeric vector, rHPIV3-
1.2HN, is demonstrated herein to induce resistance to replication of both HPIV1 and HPIV2
in hamsters. These findings illustrate the surprising flexibility of the PIV expression system.
For example, the rHPIV3-1.2HN recombinant virus contains elements from each of the three
10 serotypes of HPIV that cause significant disease: the internal genes of serotype 3 combined
with the HN and F glycoprotein genes of serotype 1, and the HN protective antigen of
serotype 2 as a supernumerary gene.

The present example provides yet another approach to deriving a PIV-based
vector vaccine to protect against both PIV1 and PIV2. In this example, the rB/HPIV3 was
15 modified by the substitution of the human PIV3 HN and F proteins by those of HPIV1.
This virus, designated rB/HPIV3.1, contains the PIV1 HN and F glycoproteins as part of the
vector backbone, intended to induce neutralizing antibodies and immunity to HPIV1. This
virus was used in the present example as a vector to express the HN and F proteins of HPIV2
singly or together as supernumerary gene(s). Three viruses were recovered and shown to be
20 fully viable: rB/HPIV3.1-2F; rB/HPIV3.1-2HN; or rB/HPIV3.1-2F,2HN, and each expressed
the PIV2 F and/or HN gene as a supernumerary gene or genes. rB/HPIV3.1-2F,2HN, which
expresses both the PIV2 F and/or HN proteins from two supernumerary genes and the PIV1
F and HN genes from the vector backbone, thus expresses both major protective antigens,
i.e., the F and HN of glycoproteins, of PIV1 and PIV2 from a single virus. This approach
25 optimizes the vaccine's protective efficacy and minimizes manufacturing costs since it
accomplishes this increased immunogenicity using only one virus. It also likely will be
simpler, safer and more effective to immunize infants and children with a single multivalent
virus compared to a mixture of several viruses.

30 Construction of antigenomic cDNAs encoding recombinant chimeric rB/HPIV3.1 viruses bearing the HPIV2 F and HN genes as additional, supernumerary genes

A full length cDNA of the BPIV3 Kansas strain in which the F and HN
glycoprotein genes of the bovine virus had been replaced with the corresponding genes of

the HPIV3 JS strain (rB/HPIV3) was constructed as previously described (Schmidt et al., J. Virol. 74:8922-9, 2000, incorporated herein by reference). This cDNA was modified to contain three additional unique restriction enzyme recognition sites (Figure 15).

Specifically, a *Bln*I site was introduced preceding the N ORF (nucleotide (nt) 103-109), an *Asc*I site (nt 1676-83) was introduced preceding the N gene end sequence and a *Not*I site (nt 3674-3681) was introduced preceding the P gene end sequence. Next, the F and HN glycoprotein genes of rB/HPIV3 were substituted with the corresponding genes of HPIV1.

To achieve this, the sub-clone 3.1hcR6 of the previously described rHPIV3-1 full length cDNA (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference), which contained the ORFs of the F and HN glycoprotein genes of HPIV1 under the control of HPIV3 transcription signals was modified by PCR mutagenesis to create a *Sgr*AI restriction enzyme recognition site preceding the F gene and a *Bsi*WI site preceding the HN gene end sequence, analogous to the position of the *Sgr*AI and *Bsi*WI sites that had been introduced previously into rB/HPIV3 (Schmidt et al., J. Virol. 74:8922-9, 2000). The mutagenic

forward primer used to create the *Sgr*AI site was (5' to 3')
CGGCCGTGACGCGTCTCCGCACCGGTGTATTAAGCCGAAGCAAA (SEQ ID NO. 34) (*Sgr*AI site underlined), and the mutagenic reverse primer was (5' to 3')
CCCGAGCACGCTTTGCTCCTAAGTTTTTATATTTCCCGTACGTCTATTGTCTGAT TGC (SEQ ID NO. 35) (*Bsi*WI site underlined). The *Sgr*AI and *Bsi*WI sites were used to replace, as a single DNA fragment, the HPIV3 F and HN genes in rB/HPIV3 with the HPIV1 F and HN genes from the modified 3.1hcR6 plasmid. This yielded the full length antigenomic cDNA pB/HPIV3.1, consisting of HPIV1 F and HN open reading frames under the control of HPIV3 transcription signals in a background that is derived from BPIV3.

In the following step, the previously described HPIV2 F and HN open reading frames (GenBank accession numbers AF213351 and AF213352) were modified for insertion into the *Not*I and *Asc*I sites, respectively, of pB/HPIV3.1 (Figure 15). The strategy was to express each PIV2 F and HN ORF as an additional, separate mRNA, and hence it was important that it be introduced into the rB/HPIV3 genome so that it was preceded by a PIV3 gene start signal and followed by a PIV3 gene end signal. The *Not*I insertion site precedes the gene end signal of the P gene (Figure 15). Hence, for insertion at this site, the HPIV2 F ORF needed to be modified by insertion of a *Not*I site and addition of a BPIV3 gene end signal, intergenic region and gene start signal at its upstream end, and a *Not*I site at its downstream end. For the HPIV2 F ORF, the forward PCR primer used was (5' to 3')
AAAATATAGCGGCCGCAAGTAAGAAAACTTAGGATTAAAGGCGGATGGATCA

CCTGCATCCAATGATAGTATGCATTTTTGTTATGTACACTGG (SEQ ID NO. 36) and the

the reverse primer was (5' to 3')

AAAATATAGCGGCCGCTTTTACTAAGATATCCCATATATGTTTCCATGATTGTTC

5 TTGGAAAAGACGGCAGG (SEQ ID NO. 37) (*NotI* site underlined, ORF translational initiation and termination triplets in bold). For the HPIV2 HN ORF, the same cis-acting elements as described above for HPIV2 F were added, but instead of *NotI*, an *AscI* site was added on either side of the insert to facilitate cloning into the N-P gene junction. The forward PCR primer used was (5' to 3')

10 GGAAAGGCGCGCCCAAAGTAAGAAAACTTAGGATTAAAGGCGGA
TGGAAGATTACAGCAATCTATCTCTTAAATCAATTCC (SEQ ID NO. 38), the reverse primer was (5' to 3')

GGAAAGGCGCGCCCAAATTAAGCATTAGTTCCTTAAAAATGGTATTATTG
(SEQ ID NO. 39).

15 The PCR products were digested with *NotI* (HPIV2 F insert) or *AscI* (HPIV2 HN insert) and cloned into the modified full length cDNA clone using standard molecular cloning techniques. The resulting full length cDNA containing the HPIV2 F ORF was designated pB/HPIV3.1-2F, the full length cDNA containing the HPIV2 HN ORF was designated pB/HPIV3.1-2HN, and the plasmid containing both the F and HN inserts was
20 designated pB/HPIV3.1-2F,2HN. The nucleotide sequence of each inserted gene was confirmed by restriction enzyme digestion and automated sequencing. All constructs were designed so that the final genome nucleotide length was a multiple of six, which has been shown to be a requirement for efficient RNA replication (Calain et al., *J. Virol.* 67:4822-30, 1993, incorporated herein by reference). The genome nucleotide length of the recovered
25 chimeric viruses is as follows: pB/HPIV3.1: 15492; pB/HPIV3.1-2HN: 17250; pB/HPIV3.1-2F: 17190; pB/HPIV3.1-2HN,2F: 18948.

Recovery of rB/HPIV3.1, rB/HPIV3.1-2F, rB/HPIV3.1-2HN, and rB/HPIV3.1-2F,2HN chimeric viruses from cDNA

30 rB/HPIV3.1, rB/HPIV3.1-2F, rB/HPIV3.1-2HN, and rB/HPIV3.1-2F,2HN chimeric viruses were recovered from the cDNAs pB/HPIV3.1, pB/HPIV3.1-2F, pB/HPIV3.1-2HN, and pB/HPIV3.1-2F,2HN, respectively. This was accomplished by the previously-described method in which HEP-2 cells were transfected with the respective

antigenomic cDNA together with BPIV3 N, P and L support plasmids. The cells were simultaneously infected with a recombinant vaccinia virus, strain MVA, expressing the T7 RNA polymerase gene. Porcine trypsin was added to the cell culture medium to activate the HPIV1 F protein, as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998). The recovered recombinant viruses were cloned biologically by sequential terminal dilution in Vero cells. All of the recombinant viruses replicated efficiently, induced CPE in Vero cells within 5 days and rendered the cell monolayer positive for hemadsorption. The presence of the inserted HPIV2 F and HN gene in the backbone of each recovered recombinant virus was confirmed by RT-PCR of viral RNA isolated from infected cells followed by restriction enzyme digestion and DNA sequencing. The sequence of the inserted gene and flanking regions in the recovered recombinant viruses was identical to that of the starting antigenomic cDNA.

EXAMPLE XII

15 Use of rHPIV3-1 cp45_L as a Vector For The Measles Virus Hemagglutinin (HA) protein: Development of a Sequential Immunization Strategy

The chimeric rHPIV3-1 virus, which has a HPIV3 backbone in which the HPIV3 HN and F genes have been replaced by their HPIV1 counterparts, was shown above to serve as a useful vector for the HPIV2 HN protein as a supernumerary gene. This chimeric vector, rHPIV3-1.2HN, was able to induce resistance to replication of both HPIV1 and HPIV2 in hamsters. This finding illustrates the surprising flexibility of the PIV expression system. For example, this particular virus, rHPIV3-1.2HN, contained elements from each of the three serotypes of HPIV: the internal genes of serotype 3 combined with the HN and F glycoprotein genes of serotype 1, and the HN protective antigen of serotype 2 as a supernumerary gene. A further derivative, rHPIV3-1.2HNcp45_L, was also made that contained attenuating mutations from the cp45 HPIV3 vaccine candidate.

Thus, a PIV vector can be represented as comprising three components: the internal vector backbone genes, which can contain attenuating mutations as desired; the vector glycoprotein genes, which can be of the same or of a heterologous serotype; and one or more supernumerary genes encoding protective antigens for additional pathogens. In most cases, these supernumerary antigens are not incorporated into the virion and hence do not change the neutralization or tropism characteristics of the virus. Thus, each PIV vector is a bivalent or multivalent vaccine in which the vector itself induces immunity against an

important human pathogen and each supernumerary antigen induces immunity against an additional pathogen.

In the present example, the flexibility of the PIV vector system is further demonstrated by using the rHPIV3-1 virus, as well as its attenuated rHPIV3-1cp45_L derivative, as vectors to express measles virus HA as a supernumerary gene. This provides a new bivalent vaccine candidate for HPIV1 and measles virus. Thus, measles virus HA can be vectored by rHPIV3 and attenuated derivatives thereof, bearing the serotype 3 antigenic determinants, or by rHPIV3-1 and attenuated derivatives thereof, bearing the serotype 1 antigenic determinants.

It is noteworthy that the three serotypes of HPIV (1, 2 and 3) do not confer significant cross-protection, and that each represents a significant human pathogen for which a vaccine is needed. This raises the possibility that the three serotypes might be used to sequentially immunize the infant against the PIVs as well as vectored protective antigens against heterologous pathogens. Specifically, immunization with a PIV vector containing the antigenic determinants of one serotype should be affected minimally or not at all by prior immunization with a vector or vectors containing the antigenic determinants of a heterologous serotype. This provides the opportunity to perform sequential immunizations and boosts (preferentially at intervals of 4-6 weeks or more) against supernumerary antigens as well as against the three HPIV serotypes, whose genes can be expressed either in the vector backbone or as supernumerary genes.

The present example details the use of the techniques of reverse genetics to develop a live-attenuated HPIV1 candidate vaccine, rPIV3-1HA_{P-M} cp45_L, expressing as a supernumerary gene the major measles virus protective antigen, the HA glycoprotein (Durbin, *J. Virol.* 74:6821-31, 2000, incorporated herein by reference), for use in infants and young children to induce an immune response against both measles virus and HPIV1. Also, a sequential immunization schedule was developed in which immunization with the attenuated rHPIV3 HA_{P-M} cp45_L candidate vaccine (bearing the serotype 3 antigenic determinants) was followed by the rHPIV3-1 HA_{P-M} cp45_L candidate vaccine (bearing the serotype 1 antigenic determinants). Hamsters immunized with these viruses developed antibodies to the HPIV3 and HPIV1 antigens present in the backbone of the vectors and also maintained high titers of antibodies to the vectored antigen, the measles virus HA expressed as a supernumerary antigen from both the HPIV3 and HPIV1 candidate vaccine viruses.

Construction of rHPIV3-1 HA_(P-M) and rHPIV3-1 HA_(P-M) cp45_L, wild type and attenuated versions of rHPIV3-1 expressing measles virus HA as a supernumerary gene.

Two full-length plasmids were constructed, pFLC HPIV3-1 HA_(P-M) and pFLC HPIV3-1 HA_(P-M) cp45_L (Figure 16) as described above (see also, Durbin, *J. Virol.* 74:6821-31, 2000; Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998; Tao et al., *J. Virol.* 72:2955-2961, 1998, each incorporated herein by reference). pFLC HPIV3-1 HA_(P-M) was constructed using the above-described pFLC HPIV3 HA_(P-M) in which the wild type measles virus Edmonston strain HA gene ORF was inserted as a supernumerary gene between the P and M genes of rHPIV3. pFLC HPIV3 HA_(P-M) was digested with *BspEI* to *SphI* and the cDNA fragment lacking the 6487 bp *BspEI* to *SphI* sequence was isolated. Next, pFLC 2G+.hc, a full-length antigenomic cDNA plasmid bearing the F and HN ORFs of PIV1 in place of those of HPIV3 (Tao et al., *J. Virol.* 72:2955-2961, 1998) was digested with *BspEI* and *SphI*, and the 6541 bp fragment (plasmid nts 4830-11371) containing the HPIV1 glycoprotein genes in the HPIV3 backbone was inserted into the *BspEI* to *SphI* window of pFLC HPIV3 HA_{P-M} to give pFLC HPIV3-1 HA_{P-M} (Figure 15). The cp45 L mutations present in the L gene ORF (point mutations encoding amino acid substitutions Ser-942 to His, Leu-992 to Phe and Thr-1558 to Ile) are the major *ts* and *att* determinants of the HPIV3 cp45 candidate vaccine (Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998) and were previously shown to confer attenuation of replication to the rHPIV3-1 cp45_L in the respiratory tract of hamsters (Tao et al., *Vaccine* 17:1100-8, 1999). The pFLC HPIV3-1 HA_{P-M} was then modified to encode these three *ts* mutations to yield pFLC HPIV3-1 HA_{P-M} cp45_L (Figure 16). This was accomplished by inserting the *SphI* to *NgoMIV* restriction endonuclease fragment of pFLC HPIV3 cp45_L (plasmid nts 11317 -15929) (Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998) into the *SphI* to *NgoMIV* window of pFLC HPIV3-1 HA_{P-M}.

Recovery of rHPIV3-1 HA_(P-M) and rHPIV3-1 HA_(P-M) cp45_L

pFLC HPIV3-1 HA_(P-M) or pFLC HPIV3-1 HA_(P-M) cp45_L was transfected separately into HEp-2 cells on six-well plates (Costar, Cambridge, MA) together with the support plasmids pTM(N), pTM(P no C), and pTM(L) and LipofectACE (Life Technologies, Gaithersburg, MD) and the cells were simultaneously infected with MVA-T7, a replication-defective vaccinia virus recombinant encoding the bacteriophage T7 polymerase protein as

previously described (Skiadopoulos et al., Vaccine 18:503-10, 1999b, incorporated herein by reference). After incubation at 32°C for four days in medium containing trypsin, the transfection harvest was passaged onto LLC-MK2 cells in a 25 cm² flask, and the cells were incubated for 5 days at 32°C. The virus recovered from the cell supernatant was further

5 passaged on LLC-MK2 monolayer cultures with trypsin at 32°C to amplify the virus. rPIV3-1 HA_{P-M} and rPIV3-1 HA_{P-M} *cp45*_L were biologically cloned by terminal dilution on LLC-MK2 monolayer cultures at 32°C as previously described (Skiadopoulos et al., Vaccine 18:503-10, 1999b). Viral suspensions derived from biologically cloned virus were amplified on LLC-MK2 monolayer cultures.

10 Viral RNA (vRNA) was isolated from biologically cloned recombinant chimeric viruses as described above. RT-PCR was performed using rHPIV3-1 HA_{P-M} or rHPIV3-1 HA_{P-M} *cp45*_L vRNA as template and specific oligonucleotide primers that spanned the HA gene insert or the *cp45* mutations in the L gene. The RT-PCR products were analyzed by restriction endonuclease digestion and partial DNA sequencing of the PCR

15 products as described above. This confirmed the presence of the measles virus HA gene inserted between the P and M genes of rHPIV3-1 and the presence of the *cp45* L gene mutations in its attenuated derivative.

Demonstration of the attenuation phenotype of rHPIV3-1 HA_(P-M) *cp45*_L in hamsters

20 Golden Syrian hamsters in groups of six were inoculated intranasally with 10⁶ TCID₅₀ of rHPIV3-1, rHPIV3-1 HA_{P-M}, rHPIV3-1 *cp45*_L, or rHPIV3-1 HA_{P-M} *cp45*_L. Four days after inoculation the lungs and nasal turbinates were harvested and titers of virus were determined as described previously (Skiadopoulos et al., Vaccine 18:503-10, 1999b). The titers are expressed as mean log₁₀ TCID₅₀/gram tissue (Table 20). The recombinant rHPIV3-

25 1 HA_{P-M} and its parent rHPIV3-1 wt replicated to comparable levels, indicating that insertion of an additional transcription unit encoding the HA gene ORF did not further attenuate this virus for hamsters. The rHPIV3-1 HA_{P-M} *cp45*_L and its rHPIV3-1 *cp45*_L parent replicated to similar levels in the upper and lower respiratory tract indicating that rHPIV3-1 HA_{P-M} *cp45*_L was satisfactorily attenuated for replication in hamsters and that the insertion of the measles

30 virus HA gene ORF did not further attenuate the chimeric rHPIV3-1 *cp45*_L parent virus.

Table 20
Replication of wild type and attenuated versions of the rPIV3-1 and rPIV3-1 HA viruses in the respiratory tract of hamsters

Virus ^a	Mean virus titer ^b (\log_{10} TCID ₅₀ /g \pm S.E.) in:	
	Nasal Turbinates	Lungs
rPIV3-1 wt	6.3 \pm 0.1	6.6 \pm 0.2
rPIV3-1 HA _{P-M}	6.0 \pm 0.1	5.7 \pm 0.7
rPIV3-1 cp45 _L	4.1 \pm 0.2	1.8 \pm 0.2
rPIV3-1 HA _{P-M} cp45 _L	4.4 \pm 0.2	1.9 \pm 0.2

a. Groups of 6 hamsters each were inoculated with 10^6 TCID₅₀ of the indicated virus intranasally.
b. Lungs and nasal turbinates were harvested four days later. Virus present in tissue homogenates was titered by serial dilution on LLC-MK2 monolayer cultures at 32°C.
Guinea pig erythrocytes were used for hemadsorption.

A sequential immunization schedule employing immunization with the attenuated rHPIV3 HA_{P-M}cp45_L chimeric vaccine candidate followed by the attenuated rHPIV3-1 HA_{P-M} cp45_L vaccine candidate induces antibodies to the HPIV3 and HPIV1 antigens of the vector backbones and induces and maintains high titers of antibodies to the shared vectored antigen, the measles virus HA.

Immunization of a group of hamsters with rHPIV3-1 HA_{P-M} cp45_L induced a strong immune response to both the HPIV1 and to the measles virus (Table 21, group 6) indicating that rHPIV3-1, like rHPIV3, can be an efficient vector for the measles virus HA.

The feasibility of sequential immunization of hamsters with rHPIV3 HA_{P-M} cp45_L and rHPIV3-1 HA_{P-M} cp45_L was next examined. Groups of hamsters were immunized with 10⁶ TCID₅₀ of rHPIV3 HA_{P-M} cp45_L (Table 21, groups 1, 2 and 3), rHPIV3 cp45_L (group 4), or L15 medium control (group 5) (Table 21). 59 days after the first immunization, groups of hamsters were immunized with 10⁶ TCID₅₀ of rHPIV3-1 HA_{P-M} cp45_L (group 1 and 4), rHPIV3-1 cp45_L (group 2 and 5), or L15 medium control (group 3). Serum samples were collected before the first immunization, 58 days after the first immunization, and 35 days after the second immunization. Animals immunized with rHPIV3 cp45_L (Table 21, group 4) developed a strong antibody response to HPIV3, and animals immunized with rHPIV3 HA_{P-M} cp45_L (groups 1, 2 and 3) developed a strong antibody response to both HPIV3 and measles virus. Animals in Group 4, which had been previously immunized with rHPIV3 cp45_L, were subsequently immunized with rHPIV3-1 HA_{P-M} cp45_L on day 59. When assayed on day 94, these animals had high titers of antibodies against HPIV3 and measles virus and a low to moderate level of antibodies to HPIV1. This showed that the HPIV3-1 chimeric vaccine virus was able to induce an immune response to both the HPIV1 antigens of the vector and to the vectored HA protein even in the presence of immunity to HPIV3, but there was some diminution of its immunogenicity in animals immune to HPIV3. The rHPIV3-1 HA_{P-M} cp45_L vaccine was clearly immunogenic in animals previously immune to HPIV3 as indicated by the response of hamsters in Group 4. These animals, which were immunized with rHPIV3 cp45_L on day 0, developed a moderately high titer of neutralizing antibodies to measles virus on day 94, 35 days following immunization with rHPIV3-1 HA_{P-M} cp45_L on day 59. Significantly, hamsters that were first immunized with rHPIV3 HA_{P-M} cp45_L and were then immunized with rHPIV3-1 HA_{P-M} cp45_L (Group 1, Table 21) achieved a higher measles virus serum neutralizing antibody titer on day 94 than groups of hamsters that were immunized with

rHPIV3 HA_{P-M} cp45_L alone (Group 3), suggesting that rHPIV3-1 HA_{P-M} cp45_L can be used to maintain high titers of serum neutralizing antibodies to measles following immunization with rHPIV3 HA_{P-M} cp45_L. Since hamsters in Group 1 developed such a high titer of antibody to the measles virus HA following first immunization with rHPIV3 HA_{P-M} cp45_L, it was not possible to detect a four-fold or greater rise of these titers following immunization with rHPIV3-1 HA_{P-M} cp45_L.

In humans, it is likely that an HPIV3 vaccine such as rHPIV3 HA_{P-M} cp45_L will be given within the first four months of life followed two months later by an HPIV1 vaccine such as rHPIV3-1 HA_{P-M} cp45_L (Skiadopoulos et al., Vaccine **18**:503-10, 1999b, incorporated herein by reference). In contrast to rodents, human infants characteristically develop low titers of antibodies to viral glycoprotein antigens administered within the first six months of life, due to immunologic immaturity, immunosuppression by maternal antibodies, and other factors (Karron et al., Pediatr. Infect. Dis. J. **14**:10-6, 1995a; Karron et al., J. Infect. Dis. **172**:1445-1450, 1995b; Murphy et al., J. Clin. Microbiol. **24**:894-8, 1986, each incorporated herein by reference). It therefore is very likely that a boosting effect of rPIV3-1 HA_{P-M} cp45_L on the antibody titers to measles virus HA will be needed and will be readily observed in those infants immunized with rPIV3 HA_{P-M} cp45_L within the first six months of life. The present example indicates that it is possible to sequentially immunize animals with two serologically distinct live attenuated PIV vaccines, each of which expresses the measles virus HA, to develop antibodies to the HPIV3 and HPIV1 antigens of the vector backbone, and to maintain high titers of antibodies to the vectored antigen, the measles virus HA.

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Table 21

Sequential immunization of hamsters with rPIV3 HA_(P-M) cp45_L followed by rPIV3-1 HA_(P-M) cp45_L induces immunity to three viruses, namely, HPIV1, HPIV3 and measles virus, and maintains the measles virus antibody titer at high levels

Group no.	Group size	Virus given in first immunization (day 0)	Immune response to first immunization			Virus given in second immunization (day 59)	Immune response to second immunization ^a		
			Serum HAI antibody titer to HPIV3 (log ₂ ± SE) ^b (day 58)	Serum antibody titer to HPIV1 ^c (log ₂ ± SE) (day 58)	Serum antibody titer to measles virus ^d (60% PRN, log ₂ ± SE) (day 58)		Serum HAI antibody titer to HPIV3 ^b (log ₂ ± SE) (day 94)	Serum neutralizing antibody titer to HPIV1 ^c (log ₂ ± SE) (day 94)	Serum antibody titer to measles virus ^d (60% PRN, log ₂ ± SE) (day 94)
1	8	rPIV3 HA _(P-M) cp45 _L	10.8±0.4	≤0.5±0.0	12.5±0.4	rPIV3-1 HA _(P-M) cp45 _L	11.5±0.5	0.9±0.2	13.1±0.3
2	8	rPIV3 HA _(P-M) cp45 _L	10.9±0.4	≤0.5±0.0	13.2±0.4	rPIV3-1 cp45 _L	10.5±0.5	1.2±0.3	12.8±0.4
3	6	rPIV3 HA _(P-M) cp45 _L	9.3±0.3	≤0.5±0.0	12.7±0.4	none	9.6±0.9	1.1±0.4	12.3±0.2
4	8	rPIV3 cp45 _L	9.6±0.6	≤0.5±0.0	< 3.3	rPIV3-1 HA _(P-M) cp45 _L	9.0±0.7	0.9±0.3	7.3±0.3
5	6	none	< 2±0.0	≤0.5±0.0	< 3.3	rPIV3-1 cp45 _L	< 2±0.0	4.8±0.6	< 3.3
6	8	rPIV3-1 HA _(P-M) cp45 _L		3.0±0.4	10.5±0.4				

a. Sera were collected 5 days before and 58 days after the first immunization. The second immunization was given 59 days after the first, and serum was collected again 35 days later (day 94).

b. Mean serum PIV3 HAI antibody titer is expressed as the reciprocal mean log₂ ± standard error, SE.

c. Mean serum neutralizing antibody titer to HPIV1 is expressed as the reciprocal mean log₂ ± S.E.

d. Mean serum neutralizing antibody titer to wild type measles virus is expressed as the reciprocal mean log₂ ± standard error, PRN, plaque reduction neutralization.

EXAMPLE XIII

Construction and Characterization of Chimeric HPIV3-2 Vaccine Recombinants Expressing Chimeric Glycoproteins

The present example details development of a live attenuated PIV2 candidate vaccine virus for use in infants and young children using reverse genetic techniques. Preliminary efforts to recover recombinant chimeric PIV3-PIV2 virus carrying full-length PIV2 glycoproteins in a wild type PIV3 backbone, as described above for HPIV3-1 chimeric constructs, did not yield infectious virus. However, viable PIV2-PIV3 chimeric viruses were recovered when chimeric HN and F ORFs rather than full-length PIV2 ORFs were used to construct the full-length cDNA. The recovered viruses, designated rPIV3-2CT in which the PIV2 ectodomain and transmembrane domain was fused to the PIV3 cytoplasmic domain and rPIV3-2TM in which the PIV2 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail domain, possessed similar, although not identical, *in vitro* and *in vivo* phenotypes. Thus, it appears that only the cytoplasmic tail of the HN or F glycoprotein of PIV3 is required for successful recovery of PIV2-PIV3 chimeric viruses.

The rPIV3-2 recombinant chimeric viruses exhibit a strong host range phenotype, i.e. they replicate efficiently *in vitro* but are strongly restricted in replication *in vivo*. This attenuation *in vivo* occurs in the absence of any added mutations from *cp45*. Although rPIV3-2CT and rPIV3-2TM replicated efficiently *in vitro*, they were highly attenuated in both the upper and the lower respiratory tract of hamsters and African green monkeys (AGMs), indicating that chimerization of the HN and F proteins of PIV2 and PIV3 itself specified an attenuation phenotype *in vivo*. A phenotype including efficient replication *in vitro* and highly restricted growth *in vivo* is greatly desired for vaccine candidates. Despite this attenuation, they were highly immunogenic and protective against challenge with PIV2 wild type virus in both species. rPIV3-2CT and rPIV3-2TM were further modified by the introduction of the 12 PIV3 *cp45* mutations located outside of the HN and F coding sequences to derive rPIV3-2CT*cp45* and rPIV3-2TM*cp45*. These derivatives replicated efficiently *in vitro* but were even further attenuated in hamsters and AGMs indicating that the attenuation specified by the glycoprotein chimerization and by the *cp45* mutations was additive. These findings identify the rPIV3-2CT and rPIV3-2TM recombinants as preferred candidates for use in live attenuated PIV2 vaccines.

Viruses and cells

The wild type PIV1 strain used in this study, PIV1/Washington/20993/1964 (PIV1/Wash64) (Murphy et al., Infect. Immun. 12:62-68, 1975, incorporated herein by reference), was propagated in LLC-MK2 cells (ATCC CCL 7.1) as previously described (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference). The PIV wild type virus, strain V9412-6, designated PIV2/V94, was isolated in qualified Vero cells from a nasal wash of a sick child in 1994 . PIV2/V94 was plaque purified three times on Vero cells before being amplified twice on Vero cells using OptiMEM without FBS. The wild type cDNA-derived recombinant PIV3/JS strain (rPIV3/JS) was propagated as previously described (Durbin et al., Virology 235:323-332, 1997, incorporated herein by reference). The modified vaccinia Ankara virus (MVA) recombinant that expresses the bacteriophage T7 RNA polymerase was generously provided by Drs. L. Wyatt and B. Moss (Wyatt et al., Virology 210:202-205, 1995, incorporated herein by reference).

HEp-2 cells (ATCC CCL 23) were maintained in MEM (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum, 50 µg/ml gentamicin sulfate, and 2 mM glutamine. Vero cells below passage 150 were maintained in serum-free medium VP-SFM (Formula No. 96-0353SA, Life Technologies) with 50 µg/ml gentamicin sulfate and 2 mM glutamine.

Virion RNA isolation, reverse transcription and PCR amplification of viral genes, and automated sequencing

To clone viral genes or to verify genetic markers of recombinant chimeric viruses, viruses were amplified on cultured cells and concentrated by polyethylene glycol precipitation as previously described (Mbiguino et al., J. Virol. Methods 31:161-170, 1991, incorporated herein by reference). Virion RNA was extracted from the virus pellet using Trizol reagent (Life Technologies) and used as template for reverse transcription (RT) with the Superscript Preamplification system (Life Technologies). The cDNA was further PCR amplified using the Advantage cDNA kit (Clontech, Palo Alto, CA). For cloning or sequencing purposes, the RT-PCR amplified DNA was purified from agarose gels using NA45 DEAE membrane as suggested by the manufacturer (Schleicher & Schuell, Keene, NH). Sequencing was performed with the dRhodamine dye terminator cycling sequencing kit (Perkin Elmer, Forster City, CA) and an ABI 310 Gene Analyzer (Perkin Elmer, Forster City, CA).

Construction of the chimeric PIV3-PIV2 antigenomic cDNAs encoding the complete PIV2 F and HN proteins or chimeric F and HN proteins containing a PIV2-derived ectodomain and PIV3-derived cytoplasmic tail domain

A DNA encoding a full-length PIV3 antigenomic RNA was constructed in which the PIV3 F and HN ORFs were replaced by their PIV2 counterparts following the strategy described previously (Tao et al., *J. Virol.* 72:2955-2961, 1998) for PIV3-PIV1. Details of this construction are presented in Figure 17. PIV2/V94 propagated in Vero cells was concentrated and virion RNA (vRNA) was extracted from the virus pellet using Trizol reagent. The F and HN ORFs of PIV2/V94 were reverse transcribed from vRNA using random hexamer primers and the SuperScript Preamplification System before being amplified by PCR using the cDNA Advantage kit and primer pairs specific to PIV2 F and HN genes, respectively (1, 2 and 3, 4; Table 22). The amplified cDNA fragment of PIV2 F ORF was digested with NcoI plus BamHI and ligated into the NcoI-BamHI window of pLit.PIV31.Fhc (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference) to generate pLit.PIV32Fhc. The BspEI site in the PIV3 full-length cDNA is unique and we planned to use it to exchange segments between cDNAs (see Figures 17-19). Therefore, a BspEI site that was found in the PIV2 F ORF was removed by site-directed mutagenesis without affecting the amino acid sequence. The cDNA fragment of PIV2 HN ORF was digested with NcoI plus HindIII and ligated into the NcoI-HindIII window of pLit.PIV31.HNhc (Tao et al., *J. Virol.* 72:2955-2961, 1998) to generate pLit.PIV32HNhc. The PIV2 ORFs in pLit.PIV32Fhc and pLit.PIV32HNhc were sequenced, and the sequence was found to be as designed. The nucleotide sequences for the PIV2 F and HN ORFs are submitted in the GenBank. pLit.PIV32Fhc and pLit.PIV32HNhc were each digested with PpuMI plus SpeI and assembled to generate pLit.PIV32hc. The 4 kb BspEI-SpeI fragment of pLit.PIV32hc was introduced into the BspEI-SpeI window of p38'ΔPIV31hc (Skiadopoulos et al., *Vaccine* 18:503-510, 1999, incorporated herein by reference) to generate p38'ΔPIV32hc. The 6.5 kb fragment, generated by BspEI and SphI digestion of p38'ΔPIV32hc, containing the PIV2 full-length F and HN ORFs was introduced into the BspEI-SphI window of pFLC.2G+.hc (Tao et al., *J. Virol.* 72:2955-2961, 1998) to generate pFLC.PIV32hc (Figure 17; Table 23 = SEQ ID NO. 60).

Table 22. Primers used in construction of PIV3-2 full-length chimeric antigenomic cDNAs

Primer No.	Gene	Direction	Position		Used in the construction or characterization of:	Sequence ^a
			Beginning	End		
1	PIV2 F	sense	PIV2 F start codon 5070 ^b	20 bp down stream 5091	pFLC.PIV32hc	gtaccATGgATCAcCTGCATCCAAT (SEQ ID NO. 40)
2	PIV2 F	antisense	PIV2 F stop codon 6732 ^b	20 bp upstream 6705 ^b	pFLC.PIV32hc	tgtggatccTAAGATATCCCATATATGTTTC (SEQ ID NO. 41)
3	PIV2 HN	sense	PIV2 HN start codon 6837 ^b	18 bp down stream 6856 ^b	pFLC.PIV32hc	gggccATGGAAAGATTACAGCAAT (SEQ ID NO. 19)
4	PIV2 HN	antisense	PIV2 HN stop codon 8558 ^b	17 bp upstream 8538 ^b	pFLC.PIV32hc	caataagcTTAAAGCATTAGTTCCC (SEQ ID NO. 20)
5	PIV2 F	sense	5069 ^c	5088 ^c	pFLC.PIV32TM	ATGCATCACCTGCATCCAAT (SEQ ID NO. 42)
6	PIV2 F	antisense	6538 ^c	6517 ^c	pFLC.PIV32TM	TAGTGAATAAAAGTGTCTTGGCT (SEQ ID NO. 43)
7	PIV2 HN	sense	6962 ^c	6985 ^c	pFLC.PIV32TM	CATGAGATAAATTCATCTTGATGTT (SEQ ID NO. 44)
8	PIV2 HN	antisense	8560 ^c	8537 ^c	pFLC.PIV32TM	agcTTAAAGCATTAGTCCCTTAA (SEQ ID NO. 45)
9	PIV3 F	sense	6539 ^c	6566 ^c	pFLC.PIV32TM	ATCATAAATTATTTTGATAAATGATCATTA (SEQ ID NO. 46)
10	PIV3 F	antisense	5068 ^c	5050 ^c	pFLC.PIV32TM	GTTCA GTGCTTGTGTT (SEQ ID NO. 47)
11	PIV3 HN	sense	8561 ^c	8587 ^c	pFLC.PIV32TM	TCATAAATTAACCATAATATGCATCAAT (SEQ ID NO. 48)

12	PIV3 HN	antisense	6961 ^c	6938 ^c	pFLC.PIV32TM	GATGGAATTAAATTAGCACTATGAT (SEQ ID NO. 49)
13	PIV2 F	sense	5069 ^d	5088 ^d	pFLC.PIV32CT	ATGCATCACCTGTCATCCAAT (SEQ ID NO. 50)
14	PIV2 F	antisense	6607 ^d	6589 ^d	pFLC.PIV32CT	GATGATGTAGGCAATCAGC (SEQ ID NO. 51)
15	PIV2 HN	sense	6887 ^d	6904 ^d	pFLC.PIV32CT	ACTGCCACAAATTCCTTGGC (SEQ ID NO. 52)
16	PIV2 HN	antisense	8536 ^d	8511 ^d	pFLC.PIV32CT	TTAAAGCAATTAGTTCCTTAAAAATG (SEQ ID NO. 53)
17	PIV3 F	sense	6620 ^d	6642 ^d	pFLC.PIV32CT	AAGTATTACAGAAATTCAAAAAGAG (SEQ ID NO. 54)
18	PIV3 F	antisense	5068 ^d	5050 ^d	pFLC.PIV32CT	GTTCAGTGCTTGTGTGT (SEQ ID NO. 47)
19	PIV3 HN	sense	8525 ^d	8551 ^d	pFLC.PIV32CT	TCATAATTAAACCATAATATGCATCAAT (SEQ ID NO. 48)
20	PIV3 HN	antisense	6898 ^d	6879 ^d	pFLC.PIV32CT	CTTATTAGTGAGCTTGTGTGC (SEQ ID NO. 55)
21	PIV2 F	Sense	6608 ^{c, d}	6630 ^{c, d}	Chimera confirmation	ACCGCAGCTGTAGCAATAGT (SEQ ID NO. 56)
22	PIV2 HN	antisense	7522 ^c 7501 ^d	7502 ^c 7481 ^d	Chimera confirmation	GATTCCATCACTTAGGTAAAT (SEQ ID NO. 57)
23	PIV3 M	sense	4759 ^{c, d}	4780 ^{c, d}	Chimera confirmation	GATACTATCCTAATATTATTGC (SEQ ID NO. 58)
24	PIV3 L	antisense	9100 ^c 9076 ^d	9081 ^c 9057 ^d	Chimera confirmation	GCTAATTTTGTAGCACATT (SEQ ID NO. 59)

^a All the primers are annotated in that the PIV specific sequences are in uppercase, non-PIV sequences in lowercase, start and stop codons in bold, and restriction sites underlined.

^b The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIIV32hc.

^c The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIIV32TM and pFLC.PIIV32TMcp45.

^d The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIIV32CT and pFLC.PIIV32CTcp45.

TABLE 23 (SEQ ID NO. 60)

Sequence of pFLC.PIV32, 15492 bp in sense orientation
(only the insert is shown)

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1 ACCAAACAAG AGAAGAAACT TGTCTGGGAA TATAAATTTA ACTTTAAATT AACTTAGGAT
61 TAAAGACATT GACTAGAAGG TCAAGAAAAG GGAAGTCTAT AATTTCAAAA ATGTTGAGCC
121 TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA
181 TCATTCCCTGG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA ATAAGTATG
241 ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTTACTAGAT AATGAGAAAC
301 AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG
361 AGCTCTACCT AACACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA TACATGATTG
421 AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAACTATGT
541 TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT
601 CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTAGACAAA GATGGAACAG
721 TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA ATCATGCGGT
781 CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAAATGAAT ACCAGCAGAA
841 ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA AGAGATGCAG
901 GTCTCGCTTC ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTTGA
961 CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA CTGTATTTAT
1021 CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTCG
1081 CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT GTACAAAATA
1141 GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG TTCCAGCTAG
1201 GACAAGCAGT AGCACGTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA GATGAACCTG
1261 GAGTGACACA CGAATCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTCAG
1321 AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA GTGAAGAGC
1381 CAGAACAATT CGAACATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA TCATCCATAA
1441 TTCAATATGC CTGGGCAGAA GGAAATAGAA GCGATGATCA GACTGAGCAA GCTACAGAAT
1501 CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG AGACTCAACG
1561 ACAAGAAGAA ACAAGCAGT CAACCACCCA CTAATCCCAC AAACAGAACA AACCAGGACG
1621 AAATAGATGA TCTGTTTAAAC GCATTTGGAA GCAACTAATC GAATCAACAT TTTAATCTAA
1681 ATCAATAATA AATAAGAAAA ACTTAGGATT AAAGAATCCT ATCATACCGG AATATAGGGT
1741 GGTAAATTTA GAGTCTGCTT GAAACTCAAT CAATAGAGAG TTGATGGAAA GCGATGCTAA
1801 AAATATCAA ATCATGGATT CTTGGGAAGA GGAATCAAGA GATAAATCAA CTAATATCTC
1861 CTCGGCCCTC AACATCATTG AATTCATACT CAGCACCGAC CCCAAGAAG ACTTATCGGA
1921 AAACGACACA ATCAACACAA GAACCCAGCA ACTCAGTGCC ACCATCTGTC AACCAGAAAT
1981 CAAACCAACA GAAACAAGTG AGAAAGATAG TGGATCAACT GACAAAAATA GACAGTCCGG
2041 GTCATCACAC GAATGTACAA CAGAAGCAAA AGATAGAAAT ATTGATCAGG AAAGTGTACA
2101 GAGAGGACCT GGGAGAAGAA GCAGCTCAGA TAGTAGAGCT GAGACTGTGG TCTCTGGAGG
2161 AATCCCCAGA AGCATCACAG ATTCTAAAAA TGGAACCCAA AACACGGAGG ATATTGATCT
2221 CAATGAAATT AGAAAGATGG ATAAGGACTC TATTGAGGGG AAAATGCGAC AATCTGCAAA
2281 TGTTCCAAGC GAGATATCAG GAAGTGATGA CATATTTACA ACAGAACAAA GTAGAAACAG
2341 TGATCATGGA AGAAGCCTGG AATCTATCAG TACACCTGAT ACAAGATCAA TAAGTGTGT
2401 TACTGCTGCA ACACCAGATG ATGAAGAAGA AATACTAATG AAAAAATAGTA GGACAAAGAA
2461 AAGTTCTTCA ACACATCAAG AAGATGACAA AAGAATTAAA AAAGGGGGAA AAGGGAAAGA
2521 CTGGTTTAAG AAATCAAAAG ATACCGACAA CCAGATACCA ACATCAGACT ACAGATCCAC
2581 ATCAAAAGGG CAGAAGAAAA TCTCAAAGAC AACAACCACC AACACCGACA CAAAGGGGCA
2641 AACAGAAATA CAGACAGAAT CATCAGAAAC ACAATCCTCA TCATGGAATC TCATCATCGA
2701 CAACAACACC GACCGGAACG AACAGACAAG CACAACCTCT CCAACAACAA CTTCCAGATC
2761 AACTTATACA AAAGAATCGA TCCGAACAAA CTCTGAATCC AAACCAAGA CACAAAAGAC
2821 AAATGGAAGG GAAAGGAAGG ATACAGAAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC
2881 TCTATTGCAG AATCTTGGTG TAATTCATC CACATCAAAA CTAGATTTAT ATCAAGACAA
2941 ACGAGTTGTA TGTGTAGCAA ATGTACTAAA CAATGTAGAT ACTGCATCAA AGATAGATTT
3001 CCTGGCAGGA TTAGTCATAG GGGTTTCAAT GGACAACGAC ACAAATTTAA CACAGATACA
3061 AAATGAAATG CTAAACCTCA AAGCAGATCT AAAGAAAATG GACGAATCAC ATAGAAGATT
3121 GATAGAAAAT CAAAGAGAAC AACTGTCATT GATCACGTCA CTAATTTCAA ATCTCAAAAT
3181 TATGACTGAG AGAGGAGGAA AGAAAGACCA AAATGAATCC AATGAGAGAG TATCCATGAT
3241 CAAAACAAAA TTGAAAGAAG AAAAGATCAA GAAGACCAGG TTTGACCCAC TTAGGAGGC
3301 ACAAGGCATT GACAAGAATA TACCCGATCT ATATCGACAT GCAGGAGATA CACTAGAGAA
3361 CGATGTACAA GTTAAATCAG AGATATTAAG TTCATACAAT GAGTCAAATG CAACAAGACT

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3421	AATACCCAAA	AAAGTGAGCA	GTACAATGAG	ATCACTAGTT	GCAGTCATCA	ACAACAGCAA
3481	TCTCTCACAA	AGCACAAAAC	AATCATACAT	AAACGAACTC	AAACGTTGCA	AAAATGATGA
3541	AGAAGTATCT	GAATTAATGG	ACATGTTCAA	TGAAGATGTC	AACAATTGCC	AATGATCCAA
3601	CAAAGAAACG	ACACCGAACA	AACAGACAAG	AAACAACAGT	AGATCAAAAC	CTGTCAACAC
3661	ACACAAAATC	AAGCAGAATG	AAACAACAGA	TATCAATCAA	TATACAAATA	AGAAAAACTT
3721	AGGATTAAAG	AATAAATTAA	TCCTTGTCCA	AAATGAGTAT	AACTAACTCT	GCAATATACA
3781	CATTCCCAGA	ATCATCATTC	TCTGAAAATG	GTCATATAGA	ACCATTACCA	CTCAAAGTCA
3841	ATGAACAGAG	GAAAGCAGTA	CCCCACATTA	GAGTTGCCAA	GATCGGAAAT	CCACCAAAAC
3901	ACGGATCCCG	GTATTTAGAT	GTCTTCTTAC	TCGGCTTCTT	CGAGATGGAA	CGAATCAAAG
3961	ACAAATACGG	GAGTGTGAAT	GATCTCGACA	GTGACCCGAG	TTACAAAGTT	TGTGGCTCTG
4021	GATCATTACC	AATCGGATTG	GCTAAGTACA	CTGGGAATGA	CCAGGAATTG	TTACAAGGCC
4081	CAACCAAACT	GGATATAGAA	GTGAGAAGAA	CAGTCAAAGC	GAAAGAGATG	GTTGTTTACA
4141	CGGTACAAAA	TATAAAACCA	GAACGTGTAC	CATGGTCCAA	TAGACTAAGA	AAGGAATATG
4201	TGTTTCGATGC	CAACAAAGTT	GCTCTTGCTC	CTCAATGTC	TCCACTAGAT	AGGAGCATAA
4261	AATTTAGAGT	AATCTTTCGTG	AATTGTACGG	CAATTGGATC	AATAACCTTG	TTCAAAATTC
4321	CTAAGTCAAT	GGCATCACTA	TCTCTACCCA	ACACAATATC	AATCAATCTG	CAGGTACACA
4381	TAAAAACAGG	GGTTCAGACT	GATTCTAAAG	GGATAGTTCA	AATTTTGGAT	GAGAAAGGCG
4441	AAAAATCACT	GAATTTTCATG	GTCCATCTCG	GATTGATCAA	AAGAAAAGTA	GGCAGAATGT
4501	ACTCTGTTGA	ATACTGTAAA	CAGAAAATCG	AGAAAATGAG	ATTGATATTT	TCTTTAGGAC
4561	TAGTTGGAGG	AATCAGTCTT	CATGTCAATG	CAACTGGGTC	CATATCAAAA	ACCATAGCAA
4621	GTCAGCTGGT	ATTCAAAGA	GAGATTGTGT	ATCCTTTAAT	GGATCTAAAT	CCGCATCTCA
4681	ATCTAGTTAT	CTGGGCTTCA	TCAGTAGAGA	TTACAAGAGT	GGATGCAATT	TTCCAACCTT
4741	CTTTACCTGG	CGAGTTCAGA	TACTATCCTA	ATATTATTGC	AAAAGGAGTT	GGGAAAATCA
4801	AACAATGGAA	CTAGTAATCT	CTATTTTAGT	CCGGACGTAT	CTATTAAGCC	GAAGCAAATA
4861	AAGGATAATC	AAAAAACTTAG	GACAAAAGAG	GTCAATACCA	ACAACATTTA	GCAGTCACAC
4921	TCGCAAGAAT	AAGAGAGAAG	GGACCAAAAA	AGTCAAATAG	GAGAAATCAA	AACAAAAGGT
4981	ACAGAACACC	AGAACAACAA	AATCAAACA	TCCAACATC	TCAAAACAAA	AATTTCAAAA
5041	GAGACCGGCA	ACACAACAAG	CATGAACAC	CATGGATCAC	CTGCATCCAA	TGATAGTATG
5101	CATTTTTGTT	ATGTACACTG	GAATTGTAGG	TTCAGATGCC	ATTGCTGGAG	ATCAACTCCT
5161	CAATGTAGGG	GTCATTCAAT	CAAAGATAAG	ATCACTCATG	TACTACACTG	ATGGTGGCGC
5221	TAGCTTTATT	GTTGTAAAA	TACTACCCAA	TCTTCCCCCA	AGCAATGGAA	CATGCAACAT
5281	CACCAAGTCTA	GATGCATATA	ATGTTACCC	ATTTAAGTTG	CTAACACCCC	TGATTGAGAA
5341	CCTGAGCAAA	ATTTCTGCTG	TTACAGATAC	CAAAACCCGC	CGAGAACGAT	TTGCAGGAGT
5401	CGTTATTGGG	CTTGCTGCAC	TAGGAGTAGC	TACAGTGC	CAATTAACCG	CAGCTGTAGC
5461	AATAGTAAAA	GCCAATGCAA	ATGCTGCTGC	GATAAACAA	CTTGATCTTT	CACTTCAATC
5521	CACCAACAAG	GCAGTATCCG	ATGTGATAAC	TGCATCAAGA	ACAATTGCAA	CCGCAGTTCA
5581	AGCGATTTCAG	GATCACAATC	ATGGAGCCAT	TGTCAACGGG	ATAACATCTG	CATCATGCCG
5641	TGCCCATGAT	GCACTAATTG	GGTCAATATT	AAATTTGTAT	CTCACTGAGC	TTACTACAAT
5701	ATTTTCATAAT	CAAATAACAA	ACCCTGCGCT	GACACCACTT	TCCATCCAAG	CTTTAAGAAT
5761	CCTCCTCGGT	AGCACCTTGC	CAATTGTCT	TGAATCCAAA	CTCAACACAA	AACTCAACAC
5821	AGCAGAGCTG	CTCAGTAGCG	GACTGTTAAC	TGGTCAAATA	ATTTCCATTT	CCCCAATGTA
5881	CATGCAAATG	CTAATTCAAA	TCAATGTTCC	GACATTTATA	ATGCAACCCG	GTCGGAAGGT
5941	AATTGATCTA	ATTGCTATCT	CTGCAAACCA	TAAATTACAA	GAAGTAGTTG	TACAAGTTCC
6001	TAATAGAATT	CTAGAATATG	CAAATGAACT	ACAAAACCTAC	CCAGCCAATG	ATTGTTTCGT
6061	GACACCAAAC	TCTGTATTTT	GTAGATACAA	TGAGGGTTCC	CCGATCCCTG	AATCACAATA
6121	TCAATGCTTA	AGGGGGAATC	TTAATTCTTG	CAC'TTTTACC	CCTATTATCG	GGAAC'TTTCT
6181	CAAGCGATTTC	GCATTTGCCA	ATGGTGTGCT	CTATGCCAAC	TGCAAACTCT	TGCTATGTAA
6241	GTGTGCCGAC	CCTCCCCATG	TTGTGCTCTA	AGATGACAAC	CAAGGCATCA	GCATAATTGA
6301	TATTAAGAGG	TGCTCTGAGA	TGATGCTTGA	CAC'TTTTTC	TTTAGGATCA	GCATCTACAT
6361	CAATGCTACA	TACGTGACAG	ACTTCTCAAT	GATTAATGCA	AATATTGTAC	ATCTAAGTCC
6421	TCTAGACTTG	TCAAATCAAA	TCAATTCAAT	AAACAAATCT	CTTAAAAGTG	CTGAGGATTG
6481	GATTGCAGAT	AGCAACTTCT	TCGCTAATCA	AGCCAGAACA	GCCAAGACAC	TTTATTCACT
6541	AAGTGCAATC	GCATTAATAC	TATCAGTGAT	TAC'TTTGGTT	GTTGTGGGAT	TGCTGATTGC
6601	CTACATCATC	AAGCTGGTTT	CTCAAATGCA	TCAATTGAGA	GCACATAGCT	CTACAACAAT
6661	GTTCCACAGG	GAGAATCCTG	CCGCTTTTTC	CAAGAACAA	CATGGAAACA	TATATGGGAT
6721	ATCTTAGGAT	CCCTACAGAT	CATTAGATAT	TAAAATTATA	AAAAACTTAG	GAGTAAAGTT
6781	ACGCAATCCA	ACTCTACTCA	TATAATTGAG	GAAGGACCCA	ATAGACAAAT	CCAAATCCAT
6841	GGAAGATTAC					

7101	TGCTTTAAAA	GATATGCACA	CCGGGAGTAT	GTCCAATGCC	AAC7GCACGC	CAGGAAATCT
7201	GCTTCTGCA7	GATGCAGCAT	ACATCAATGG	AATAAACAAA	TTCCTTG7AC	TTGAATCATA
7261	CAATGGGACG	CCTAAATATG	GACCTCTCCT	AAATATACCC	AGCTTTATCC	CCTCAGCAAC
7321	ATCTCCCCAT	GGGTGTACTA	GAATACCATC	ATTTTCACTC	ATCAAGACCC	ATTGGTGT7A
7381	CACTCACAAT	GTAATGCTTG	GAGATTGTCT	TGATTTACAG	GCATCTAACC	AGTATTTATC
7441	AATGGGGATA	ATACAACAAT	CTGCTGCAGG	GTTTCCAATT	TTCAGGACTA	TGAAAACCAT
7501	TTACCTAAGT	GATGGAATCA	ATCGCAAAAG	CTGTTCAGTC	ACTGCTATAC	CAGGAGGTTG
7561	TGTCTTG7AT	TGCTATGTAG	CTACAAGGTC	TGAAAAAGAA	GATTATGCCA	CGAGTGATCT
7621	AGCTGAAC7G	AGACTTGCTT	TCTATTATTA	TAATGATACC	TTTATTGAAA	GAGTCATATC
7681	TCTTCCAAAT	ACAACAGGGC	AGTGGGCCAC	AATCAACCCT	GCAGTCGGAA	GCGGGATCTA
7741	TCATCTAGGC	TTTATCTTAT	TTCTGTATA	TGGTGGTCTC	ATAAA7GGGA	CTACTTCTTT
7801	CAATGAGCAG	TCCTCACGCT	ATTTTATCCC	AAAACATCCC	AACATAACTT	GTGCCGG7TA
7861	CTCCAGCAAA	CAGGCTGCAA	TAGCACGGAG	TTCTATGTCT	ATCCGTTATC	ACTCAAACAG
7921	GTTAATTCTG	AGTGCTGTTC	TTATTTGTCC	ATTGCTGTGAC	ATGCATACAG	AAGAGTG7TA
7981	TCTAGTTATG	TTTACAATT	CCCAAGTCAT	GATGGGTGCA	GAAGGTAGGC	TCTATGTTAT
8041	TGGTAATAAT	TTGTATTATT	ATCAACGCAG	TTCTCTTG	TGGTCTGCAT	CGCTCTTTTA
8101	CAGGATCAAT	ACAGATTTTT	CTAAAGGAAT	TCCTCCGATC	ATTGAGGCTC	AATGGGTACC
8161	GTCCTATCAA	GTTCTCTGTC	CTGGAGTCAT	GCCATGCAAT	GCAACAAGTT	TTTGCCCTGC
8221	TAATTGCATC	ACAGGGGTGT	ACGCAGATGT	GTGGCCGCTT	AATGATCCAG	AACTCATGTC
8281	ACGTAATGCT	CTGAACCCCA	ACTATCGATT	TGCTGGAGCC	TTTCTCAAAA	ATGAGTCCAA
8341	CCGAAC7AAT	CCCACTATT	ACACTGCATC	GGCTAACTCC	CTCTTAAATA	CTACCGGATT
8401	CAACAACACC	AATCAGAAAT	CAGCATATAC	ATCTTCAACC	TGCTTTAAAA	ACACTGGAAC
8461	CCAAAAAATT	TATTGTTTAA	TAATAATTGA	AATGGGCTCA	TCTCTTTTAG	GGGAGTTCCA
8521	AATAATACCA	TTTTTAAAGG	AACTAATGCT	T7AAGCTTAA	TTAACCATAA	TATGCATCAA
8581	TCTATCTATA	ATACAAGTAT	ATGATAAGTA	ATCTGCAATC	AGACAATAGA	CAAAAGGGAA
8641	ATATAAAAAA	CTTAGGAGCA	AAGCGTGCTC	GGGAAATGGA	CACTGAATCT	AACAATGGCA
8701	CTGTATCTGA	CATACTCTAT	CCTGAGTGTC	ACCTTAACTC	TCCTATCGTT	AAAGGTAAAA
8761	TAGCACAATT	ACACACTATT	ATGAGTCTAC	CTCAGCCTTA	TGATATGGAT	GACGACTCAA
8821	TACTAGTTAT	CACTAGACAG	AAAATAAAAC	T7AATAAATT	GGATAAAAAG	CAACGATCTA
8881	TTAGAAGATT	AAAATTAAAT	TTAAGTGAAA	AAGTGAATGA	CTTAGGAAAA	TACACATTTA
8941	TCAGATATCC	AGAAATGTCA	AAAGAAATGT	TCAAATTATA	TATACTGGT	ATTAACAGTA
9001	AAGTGACTGA	ATTATTACTT	AAAGCAGATA	GAACATATAG	TCAAATGACT	GATGGATTAA
9061	GAGATCTATG	GATTAATGTG	CTATCAAAAT	TAGCCTCAAA	AAATGATGGA	AGCAATTATG
9121	ATCTTAATGA	AGAAATTAAT	AATATATCGA	AAGTTCACAC	AACCTATAAA	TCAGATAAAT
9181	GGTATAATCC	ATTCAAAACA	TGGTTTACTA	TCAAGTATGA	TATGAGAAGA	TTACAAAAAG
9241	CTCGAAATGA	GATCACTTTT	AATGTTGGGA	AGGATTATAA	CTTGTTAGAA	GACCAGAAGA
9301	ATTTCTTAT	GATACATCCA	GAATTGGTTT	TGATATTAGA	TAAACAAAAC	TATAATGGTT
9361	ATCTAATTAT	TCCTGAATTA	GTATTGATGT	ATTGTGACGT	ATTCGAAGGC	CGATGGAATA
9421	TAAGTAGCATG	TGCTAAGTTA	GATCCAAAT	TACAATCTAT	GTATCAGAAA	GGTAATAACC
9481	TGTGGGAAGT	GATAGATAAA	TTGTTTCCAA	TTATGGGAGA	AAAGACATTT	GATGTGATAT
9541	CGTTATTAGA	ACCACTTGCA	TTATCCTTAA	TTCAAAC7CA	TGATCCTGTT	AAACAAC7TA
9601	GAGGAGCTTT	TTTAAATCAT	GTGTTATCCG	AGATGGAATT	AATATTTGAA	TCTAGAGAAT
9661	CGATTAAGGA	ATTTCTGAGT	G7AGATTACA	TTGATAAAAT	TTTAGATATA	TTTAATAAGT
9721	CTACAATAGA	TGAAATAGCA	GAGATTTTCT	CTTTTTTTAG	AACATTTGGG	CATCCTCCAT
9781	TAGAAGCTAG	TATTGCAGCA	GAAAAGGTTA	GAAAATATAT	GTATATTGGA	AAACAATTAA
9841	AATTTGACAT	TATTAATAAA	TGTCATGCTA	TCTTCTGTAC	AATAATAATT	AACGGATATA
9901	GAGAGAGGCA	TGGTGGACAG	TGGCCTCCTG	TGACATTACC	TGATCATGCA	CACGAATTCA
9961	TCATAAATGC	TTACGGTTCA	AACTCTGCGA	TATCATATGA	AAATGCTGTT	GATTATTACC
10021	AGAGCTTTAT	AGGAATAAAA	TTCAATAAAT	TCATAGAGCC	TCAGTTAGAT	GAGGATTTGA
10081	CAATTTATAT	GAAAGATAAA	GCATTATCTC	CAAAAAAATC	AAATTGGGAC	ACAGTTTATC
10141	CTGCATCTAA	TTTACTGTAC	CGTACTAACG	CATCCAACGA	ATCACGAAGA	TTAGTTGAAG
10201	TATTTATAGC	AGATAGTAA	TTTGATCCTC	ATCAGATATT	GGATTATGTA	GAATCTGGGG
10261	ACTGGTTAG	TGATCCAGAA	TTTAATATTT	CTTATAGTCT	TAAAGAAAAA	GAGATCAAAC
10321	AGGAAGGTAG	ACTCTTTGCA	AAAATGACAT	CAAAAATGAG	AGCTACACAA	GTTTTATCAG
10381	AGACCCTACT	TGCAAA7AAC	ATAGGAAAAT	TCTTTCAAGA	AAATGGGATG	GTGAAGGGAG
10441	AGATTGAATT	ACTTAAGAGA	TTAACAACCA	TATCAATATC	AGGAGTTCCA	CGGTATAATG
10501	AAGTGTACAA	TAATTCTAAA	AGCCATACAG	ATGACCTTAA	AACCTACAAT</	

10861	GTTTTTACGT	TCATAACCCA	AGAGGGGGTA	TAGAAGGATT	TTGTCAAAAA	TTATGGACAC
10921	TCATATCTAT	AAGTGAATA	CATCTAGCAG	CTGTTAGAAT	AGGCGTGAGG	GTGACTGCAA
10981	TGGTTCAAGG	AGACAATCAA	GCTATAGCTG	TAACCACAAG	AGTACCCAAC	AATTATGACT
11041	ACAGAGTTAA	GAAGGAGATA	GTTTATAAAG	ATGTAGTGAG	ATTTTTTTGAT	TCATTAAGAG
11101	AAGTATGGA	TGATCTAGGT	CATGAACCTA	AATTAAGATG	AACGATTATA	AGTAGCAAGA
11161	TGTTCATATA	TAGCAAAAAG	ATCTATTATG	ATGGGAGAAT	TCTTCTCAA	GCTCTAAAAG
11221	CATTATCTAG	ATGTGTTCTC	TGGTCAGAGA	CAGTAATAGA	CGAAACAAGA	TCAAGATCTT
11281	CAAATTTGGC	AACATCATTT	GCAAAAGCAA	TTGAGAATGG	TTATTCACCT	GTTCTAGGAT
11341	ATGCATGCTC	AATTTTTAAG	AATATTCAAC	AACTATATAT	TGCCCTTGGG	ATGAATATCA
11401	ATCCAACAT	AACACAGAAT	ATCAGAGATC	AGTATTTTAG	GAATCCAAAT	TGGATGCAAT
11461	ATGCCTCTTT	AATACCTGCT	AGTGTTGGGG	GATTCAATTA	CATGGCCATG	TCAAGATGT
11521	TTGTAAGGAA	TATTGGTGAT	CCATCAGTTG	CCGCATTGGC	TGATATTAAA	AGATTTTATTA
11581	AGGCGAATCT	ATTAGACCGA	AGTGTTCTTT	ATAGGATTAT	GAATCAGAAA	CCAGGTGAGT
11641	CATCTTTTTT	GGACTGGGCT	TCAGATCCAT	ATTCAGCAA	TTTACCACAA	TCTCAAAATA
11701	TAACCACCAT	GATAAAAAAT	ATAACAGCAA	GGAATGTATT	ACAAGATTCA	CCAAATCCAT
11761	TATTATCTGG	ATTATTCACA	AATACAATGA	TAGAAGAAGA	TGAAGAATTA	GCTGAGTTCC
11821	TGATGGACAG	GAAGGTAATT	CTCCCTAGAG	TTGCACATGA	TATTCTAGAT	AATTCTCTCA
11881	CAGGAATTAG	AAATGCCATA	GCTGGAATGT	TAGATACGAC	AAAATCACTA	ATTCGGGTTG
11941	GCATAAATAG	AGGAGGACTG	ACATATAGTT	TGTTGAGGAA	AATCAGTAAT	TACGATCTAG
12001	TACAATATGA	AACACTAAGT	AGGACTTTTG	GACTAATTGT	AAGTGATAAA	ATCAAGTATG
12061	AAGATAATGTG	TTCGGTAGAC	CTTGCCATAG	CATTGCGACA	AAAGATGTGG	ATTCATTTAT
12121	CAGGAGGAAG	GATGATAAGT	GGACTTGAAA	CGCCTGACCC	ATTAGAATTA	CTATCTGGGG
12181	TAGTAATAAC	AGGATCAGAA	CATTGTAAAA	TATGTTATTC	TTCAGATGGC	ACAAACCCAT
12241	ATACTTGGAT	GTATTTACCC	GGTAATATCA	AAATAGGATC	AGCAGAAACA	GGTATATCGT
12301	CATTAAGAGT	TCCTTATTTT	GGATCAGTCA	CTGATGAAAG	ATCTGAAGCA	CAATTAGGAT
12361	ATATCAAGAA	TCCTTAGTAAA	CCTGCAAAAG	CCGCAATCAG	AATAGCAATG	ATATATACAT
12421	GGGCAATTTG	TAATGATGAG	ATATCTTTGA	TGGAAGCCTC	ACAGATAGCA	CAAAACCGTG
12481	CAAAATTTTAC	ACTAGATAGT	CTCAAAATTT	TAACACCGGT	AGCTACATCA	ACAAATTTAT
12541	CACACAGATT	AAAGGATACT	GCAACTCAGA	TGAAATTCTC	CAGTACATCA	TTGATCAGAG
12601	TCAGCAGATT	CATAACAATG	TCCAATGATA	ACATGTCTAT	CAAAGAAGCT	AATGAAACCA
12661	AAGATACTAA	TCTTATTTAT	CAACAAATAA	TGTTAACAGG	ATTAAGTGTT	TTCGAATATT
12721	TATTTAGATT	AAAAGAAACC	ACAGGACACA	ACCCTATAGT	TATGCATCTG	CACATAGAAG
12781	ATGAGTGTTG	TATTAAGAA	AGTTTTAATG	ATGAACATAT	TAATCCAGAG	TCTACATTAG
12841	AAGTAATTCG	ATATCCTGAA	AGTAATGAAT	TTATTTATGA	TAAAGACCCA	CTCAAGATG
12901	TGGACTTATC	AAAACCTTAG	GTTATTAAG	ACCATTCTTA	CACAATTGAT	ATGAATTATT
12961	GGGATGATAC	TGACATCATA	CATGCAATTT	CAATATGTAC	TGCAATTACA	ATAGCAGATA
13021	CTATGTCACA	ATTAGATCGA	GATAATTTAA	AAGAGATAAT	AGTTATTGCA	AATGATGATG
13081	ATATTAATAG	CTTAATCACT	GAATTTTTGA	CTCTTGACAT	ACTTGTATTT	CTCAAGACAT
13141	TTGGTGGAAT	ATTAGTAAAT	CAATTTGCA	ACACTCTTTA	TAGTCTAAAA	ATAGAAGGTA
13201	GGGATCTCAT	TTGGGATTAT	ATAATGAGAA	CACGTGAGAA	TACTTCCCAT	TCAATATTAA
13261	AAGTATCTAT	TAATGCTATTA	TCTCATCTTA	AAGTATTCAA	GAGGTTCTGG	GATTGTGGAG
13321	TTTTAAACCC	TATTTATGGT	CCTAATACTG	CTAGTCAAGA	CCAGATAAAA	CTTGCCCTAT
13381	CTATATGTGA	ATATTCACCTA	GATCTATTTA	TGAGAGAATG	GTTGAATGGT	GTATCACTTG
13441	AAATATACAT	TTGTGACAGC	GATATGGAAG	TTGCAAAATG	TAGGAAACAA	GCCTTTATTT
13501	CTAGACACCT	TTCAATTGTT	TGTTGTTTAG	CAGAAATTGC	ATCTTTTCGGA	CCTAACCTGT
13561	TAAACTTAAC	ATACTTGGAG	AGACTTGATC	TATTGAAACA	ATATCTTGAA	TTAAATATTA
13621	AAGAAGACCC	TACTCTTAA	TATGTACAAA	TATCTGGATT	ATTAATTTAA	TGCTTCCCAT
13681	CACCTGTAAC	ATACGTAGA	AAGACTGCAA	TCAAATATCT	AAGGATTGCG	GGTATTAGTC
13741	CACCTGAGGT	AATTGTAGAT	TGGGATCCGG	TAGAAGATGA	AAATATGCTG	GATAACATTG
13801	TCAAACTAT	AAATGATAAC	TGTAATAAAG	ATAATAAAGG	GAATAAAATT	AACAATTTCT
13861	GGGGACTAGC	ACTTAAGAAC	TATCAAGTCC	TTAAAATCAG	ATCTATAACA	AGTGATTCTG
13921	ATGATAATGA	TAGACTAGAT	GCTAATACAA	GTGGTTTGAC	ACTTCCTCAA	GGAGGGAATT
13981	ATCTATCGCA	TCAATTGAGA	TTATTTCGAA	TCAACAGCAC	TAGTTGTCTG	AAAGCTCTTG
14041	AGTTATCACA	AATTTTAATG	AAGGAAGTCA	ATAAAGACAA	GGACAGGCTC	TTCTTGCGAG
14101	AAGGAGCAGG	AGCTATGCTA	GCATGTTATG	ATGCCACATT	AGGACCTGCA	GTTAATTATT
14161	ATAATTCAGG	TTTGAATATA	ACAGATGTAA	TTGGTCAACG	AGAATTGAAA	ATATTTCTTT
14221	CAGAGGTATC					

14581	TCAAACTTC	TAATCCTGCA	TCAACAGAAT	TATATCTAAT	TCGAAAGAT	GCATATTGTA
14641	CTATAATGGA	ACCTAGTGAA	ATTGTTTTAT	CAAACTTAA	AAGATTGTCA	CTCTTGGGAA
14701	AAAATAATCT	ATTAAAATGG	ATCATTTTAT	CAAAGAAGAG	GAATAATGAA	TGGTTACATC
14761	ATGAAATCAA	AGAAGGAGAA	AGAGATTATG	GAATCATGAG	ACCATATCAT	ATGGCACTAC
14821	AAATCTTTGG	ATTTCAAATC	AATTTAAATC	ATCTGGCGAA	AGAATTTTAA	TCAACCCCAG
14881	ATCTGACTAA	TATCAACAAT	ATAATCCAAA	GTTTTAGCG	AACAATAAAG	GATGTTTTAT
14941	TTGAATGGAT	TAATATAACT	CATGATGATA	AGAGACATAA	ATTAGGCGGA	AGATATAACA
15001	TATTCCCACT	GAAAAATAAG	GGAAAGTTAA	GAATGCTATC	GAGAAGACTA	GTATTAAGTT
15061	GGATTTTATT	ATCATTATCG	ACTCGATTAC	TTACAGGTCG	CTTTCCTGAT	GAAAAATTTG
15121	AACATAGAGC	ACAGACTGGA	TATGTATCAT	TAGCTGATAC	TGATTTAGAA	TCATTAAAGT
15181	TATTGTGCGA	AAACATCATT	AAGAATTACA	GAGAGTGTAT	AGGATCAATA	TCATATTGGT
15241	TTCTAACCAA	AGAAGTTAAA	ATACTTATGA	AATTGATCGG	TGGTGCTAAA	TTATTAGGAA
15301	TTCCAGACA	ATATAAAGAA	CCCGAAGACC	AGTTATTAGA	AACTACAAT	CAACATGATG
15361	AATTTGATAT	CGATTAAAAC	ATAAATACAA	TGAAGATATA	TCCTAACCTT	TATCTTTAAG
15421	CCTAGGAATA	GACAAAAAGT	AAGAAAAACA	TGTAATATAT	ATATACCAAA	CAGAGTTCTT
15481	CTCTTGTTTG	GT				

In a second strategy (Figure 18), chimeric PIV3-PIV2 F and HN ORFs rather than the complete ORF exchange were constructed in which regions of the PIV2 F and HN ORFs encoding the ectodomains were amplified from pLit.PIV32Fhc and pLit.PIV32HNhc, respectively, using PCR, Vent DNA polymerase (NEB, Beverly, MA), and primer pairs
5 specific to PIV2 F (5, 6 in Table 22) and HN (7, 8 in Table 22). In parallel, the regions of PIV3 F and HN ORFs encoding the ectodomains were deleted from their cDNA subclones pLit.PIV3.F3a and pLit.PIV3.HN4 (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), respectively, using PCR, Vent DNA polymerase, and primer pairs
10 cDNA fragments of PIV2 and PIV3 were purified from agarose gels and ligated to generate pLit.PIV32FTM and pLit.PIV32HNTM, respectively. The chimeric F and HN constructs were digested with PpuMI plus SpeI and assembled together to generate pLit.PIV32TM, which was subsequently sequenced with the dRhodamine dye terminator sequencing kit across its PIV specific region in its entirety and found to be as designed. The 4 kb BspEI-SpeI
15 fragment from pLit.PIV32TM was then introduced into the BspEI-SpeI window of p38'ΔPIV31hc to generate p38'ΔPIV32TM. The 6.5 kb BspEI-SphI fragment from p38'ΔPIV32TM, containing the PIV3-PIV2 chimeric F and HN genes, was introduced into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45 (Skiadopoulos et al., *J. Virol.* 73:1374-81, 1999, incorporated herein by reference) to generate pFLC.PIV32TM (Table 24; SEQ ID NO. 61) and pFLC.PIV32TMcp45, respectively. The nucleotide sequence of the
20 BspEI-SpeI fragment, containing the chimeric PIV3-PIV2 F and HN genes, is submitted in the GenBank.

TABLE 24 (SEQ ID NO. 61)

Sequence of pFLC.PIV32TM, 15498 bp in sense orientation
(only the antigenome is shown)

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1 ACCAAACAAG AGAAGAAACT TGTCTGGGAA TATAAATTTA ACTTTAAATT AACTTAGGAT
61 TAAAGACATT GACTAGAAGG TCAAGAAAAG GGAACCTCTAT AATTTCAAAA ATGTTGAGCC
121 TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA
181 TCATTCTCTG ACAGAAAAAT ACTGCTCTCTA TATTCGCCCT TGGACCGACA ATAAGTATG
241 ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT AATGAGAAAC
301 AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG
361 AGCTCTACCT AACAACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA TACATGATTG
421 AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAACTATGT
541 TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT
601 CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTGAGACAA GATTGGAACG
721 TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA ATCATGCGGT
781 CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAATGAAT ACCAGCAGAA
841 ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA AGAGATGCAG
901 GTCTCGCTTC ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTTGA
961 CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA CTGTATTTAT
1021 CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTCT
1081 CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT GTACAAAATA
1141 GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG TTCCAGCTAG
1201 GACAAGCAGT AGCAGCTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA GATGAACTTG
1261 GAGTGACACA CGAATCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTCAG
1321 AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA GATGAAGAGC
1381 CAGAACAATT CGAATATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA TCATCCATAA
1441 TTCAATATGC CTGGGCAGAA GGAAATAGAA GCGATGATCA GACTGAGCAA GCTACAGAAT
1501 CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG AGACTCAACG
1561 ACAAGAAGAA ACAAGCAGT CAACCACCCA CTAATCCCAC AAACAGAACA AACCAGGACG
1621 AAATAGATGA TCTGTTTAAC GCATTTGGAA GCAACTAATC GAATCAACAT TTTAATCTAA
1681 ATCAATAATA AATAAGAAAA ACTTAGGATT AAAGAATCCT ATCATACCGG AATATAGGGT
1741 GGTAAATTTA GAGTCTGCTT GAAACTCAAT CAATAGAGAG TTGATGGAAA GCGATGCTAA
1801 AAATATCAA ATCATGGATT CTTGGGAAGA GGAATCAAGA GATAAATCAA CTAATATCTC
1861 CTCGGCCCTC AACATCATTG AATTCATACT CAGCACCGAC CCCCAGAAG ACTTATCGGA
1921 AAACGACACA ATCAACACAA GAACCCAGCA ACTCAGTGCC ACCATCTGTC AACCAGAAAT
1981 CAAACCAACA GAAACAAGTG AGAAAGATAG TGGATCAACT GACAAAATA GACAGTCCGG
2041 GTCATCACAC GAATGTACAA CAGAAGCAAA AGATAGAAAT ATTGATCAGG AAATCTGACA
2101 GAGAGGACCT GGGAGAAGAA GCAGCTCAGA TAGTAGAGCT GAGACTGTGG TACTCTGGAGG
2161 AATCCCCAGA AGCATCACAG ATTCTAAAAA TGGAACCCAA AACACGGAGG ATATTGATCT
2221 CAATGAAATT AGAAAGATGG ATAAGGACTC TATTGAGGGG AAAATGCGAC AATCTGCAAA
2281 TGTTCCAAGC GAGATATCAG GAAGTGATGA CATATTTACA ACAGAACAAA GTAGAAACAG
2341 TGATCATGGA AGAAGCCTGG AATCTATCAG TACACCTGAT ACAAGATCAA TAAGTGTGTG
2401 TACTGCTGCA ACACCAGATG ATGAAGAAGA AATACTAATG AAAAAAGTA GGACAAAGAA
2461 AAGTTCTTCA ACACATCAAG AAGATGACAA AAGAATTTAA AAAGGGGGAA AAGGGAAAGA
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2761 AACTTATACA AAAGAATCGA TCCGAACAAA CTCTGAATCC AAACCAAGA CACAAAAGAC
2821 AAATGGAAAG GAAAGGAAGG ATACAGAAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC
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3061 AAATGAAATG CTAAACCTCA AAGCAGATCT AAAGAAAATG GACGAATCAC ATAGAAGATT
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3181 TATGACTGAG AGAGGAGGAA AGAAAGACCA AAATGAATCC AATGAGAGAG TATCCATGAT
3241 CAAAACAAA TTGAAAGAAG AAAAGATCAA GAAGACCAGG TTTGACCCAC TTATGGAGGC
3301 ACAAGGCATT GACAAGAATA TACCCGATCT ATATCGACAT GCAGGAGATA CACTAGAGAA

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3361 CGATGTACAA GTTAAATCAG AGATATTAAG TTCATACAAT GAGTCAAATG CAACAAGACT
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3601 CAAAGAAACG ACACCGAACA AACAGACAAG AAACAACAGT AGATCAAAAC CTGTCAACAC
3661 ACACAAAATC AAGCAGAATG AAACAACAGA TATCAATCAA TATACAAATA AGAAAACTT
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4081 CAACCAAACCT GGATATAGAA GTGAGAAGAA CAGTCAAAGC GAAAGAGATG GTTGTTTACA
4141 CGGTACAAAA TATAAAACCA GAACTGTACC CATGGTCCAA TAGACTAAGA AAAGGAATGC
4201 TGTTTCGATGC CAACAAAGTT GCTCTTGCTC CTCAATGTCT TCCACTAGAT AGGAGCATAA
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4741 CTTTACCTGG CGAGTTCAGA TACTATCTTA ATATTATTGC AAAAGGAGTT GGGAAAATCA
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5581 GATTTCAGGAT CACATCAATG GAGCCATTGT CAACGGGATA ACATCTGCAT CATGCCGTGC
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5761 CCTCGGTAGC ACCTTGCCAA TTGTCATTGA ATCCAAACTC AACACAAAAC TCAACACAGC
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6001 TAGAATTCTA GAATATGCAA ATGAACTACA AAATAACCCA GCCAATGATT GTTTCGTGAC
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6121 ATGCTTAAGG GGAATCTTA ATTCTTGAC TTTTACCCCT ATTATCGGGA ACTTTCTCAA
6181 GCGATTGCA TTTGCCAATG GTGTGCTCTA TGCCAAGTGC AAATCTTTGC TATGTAAGTG
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6361 TGCTACATAC GTGACAGACT TCTCAATGAT TAATGCAAAT ATTGTACATC TAAGTCCTCT
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6901 ATGGACAATA ATCCTGGTGT TATTATCAAT AGTCTTCATC ATAGTGCTAA TTAATTCCAT
6961 CCATGAGATA ATTCATCTTG ATGTTTCCTC TGGTCTTATG AATTCTGATG AGTCACAGCA
7021 AGGCATTATT CAGCCTATCA TAGAATCATT AAAATCATTG ATTGCTTTGG CCAACCAGAT

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7081 TCTATATAAT GTTGCAATAG TAATTCCTCT TAAAAATTGAC AGTATCGAAA CTGTAATACT
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7201 TCTGCTTCTG CATGATGCAG CATACATCAA TGGAATAAAC AAATTCCTTG TACTTGAATC
7261 ATACAATGGG ACGCCTAAAT ATGGACCTCT CCTAAATATA CCCAGCTTTA TCCCCTCAGC
7321 AACATCTCCC CATGGGTGTA CTAGAAATACC ATCATTTTCA CTCATCAAGA CCCATTGGTG
7381 TTACACTCAC AATGTAATGC TTGGAGATTG TCTTGATTTC ACGGCATCTA ACCAGTATTT
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8221 TGCTAATTGC ATCACAGGGG TGTACGCAGA TGTGTGGCCG CTTAATGATC CAGAACTCAT
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9121 ATTATGATCT TAATGAAGAA ATTAATAATA TATCGAAAGT TCACACAACC TATAATCAG
9181 ATAAATGGTA TAATCCATTC AAAACATGGT TTACTATCAA GTATGATATG AGAAGATTAC
9241 AAAAAGCTCG AAATGAGATC ACTTTTAATG TTGGGAAGGA TTATAACTTG TTAGAAGACC
9301 AGAAGAATTT CTTATTGATA CATCCAGAAAT TGGTTTTGAT ATTAGATAAA CAAAACATA
9361 ATGGTTATCT AATTACTCCT GAATTAGTAT TGATGTATTG TGACGTAGTC GAAGGCCGAT
9421 GGAATATAAG TGCATGTGCT AAGTTAGATC CAAAATTACA ATCTATGTAT CAGAAAGGTA
9481 ATAACCTGTG GGAAGTGATA GATAAATTGT TTCCAATTAT GGGAGAAAAG ACATTTGATG
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9721 ATAAGTCTAC AATAGATGAA ATAGCAGAGA TTTTCTCTTT TTTTAGAACA TTTGGGCATC
9781 CTCCATTAGA AGCTAGTATT GCAGCAGAAA AGGTTAGAAA ATATATGTAT ATTGGAAAAC
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10081 ATTTGACAAT TTATATGAAA GATAAAGCAT TATCTCCAAA AAAATCAAAT TGGGACACAG
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10441 AGGGAGAGAT TGAATTACTT AAGAGATTAA CAACCATATC AATATCAGGA GTTCCACGGT
10501 ATAATGAAGT GTACAATAAT TCTAAAAGCC ATACAGATGA CCTTAAAACC TACAATAAAA
10561 TAAGTAATCT TAATTTGTCT TCTAATCAGA AATCAAAGAA ATTTGAATTC AAGTCAACGG
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10681 ACTGTCTTAA TTGGAGATAT GAATCAACAG CTCTATTTGG AGAAACTTGC AACCAAATAT
10741 TTGGATTAAA TAAATTGTTT AATTGGTTAC ACCCTCGTCT TGAAGGAAGT ACAATCTATG

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10801 TAGGTGATCC TTACTGTCCT CCATCAGATA AAGAACATAT ATCATTAGAG GATCACCCCTG
10861 ATTCTGGTTT TTACGTTTCAT AACCCAAGAG GGGGTATAGA AGGATTTTGT CAAAAATTAT
10921 GGACACTCAT ATCTATAAGT GCAATACATC TAGCAGCTGT TAGAATAGGC GTGAGGGTGA
10981 CTGCAATGGT TCAAGGAGAC AATCAAGCTA TAGCTGTAAC CACAAGAGTA CCCAACAATT
11041 ATGACTACAG AGTTAAGAAG GAGATAGTTT ATAAAGATGT AGTGAGATTT TTTGATTTCAT
11101 TAAGAGAAGT GATGGATGAT CTAGGTCATG AACTTAAATT AAATGAAACG ATTATAAGTA
11161 GCAAGATGTT CATATATAGC AAAAGAATCT ATTATGATGG GAGAATCTCT CCTCAAGCTC
11221 TAAAAGCATT ATCTAGATGT GTCTTCTGGT CAGAGACAGT AATAGACGAA ACAAGATCAG
11281 CATCTTCAAA TTTGGCAACA TCATTTGCAA AAGCAATTGA GAATGGTTAT TCACCTGTTT
11341 TAGGATATGC ATGCTCAATT TTTAAGAATA TTCAACAACAT ATATATTGCC CTTGGGATGA
11401 ATATCAATCC AACTATAACA CAGAATATCA GAGATCAGTA TTTTAGGAAT CCAAATTGGA
11461 TGCAATATGC CTCTTTAATA CCTGCTAGTG TTGGGGGATT CAATTACATG GCCATGTCAA
11521 GATGTTTTGT AAGGAATATT GGTGATCCAT CAGTTGCCGC ATTGGCTGAT ATTTAAAGAT
11581 TTATTAAGGC GAATCTATTA GACCGAAGTG TTCTTTATAG GATTATGAAT CAAGAACCAG
11641 GTGAGTCATC TTTTTTGGAC TGGGCTTCAG ATCCATATTC ATGCAATTTA CCACAATCTC
11701 AAAATATAAC CACCATGATA AAAAATATAA CAGCAAGGAA TGTATTACAA GATTCACCAA
11761 ATCCATTATT ATCTGGATTA TTCACAAATA CAATGATAGA AGAAGATGAA GAATTAGCTG
11821 AGTTCCTGAT GGACAGGAAG GTAATTTCTCC CTAGAGTTGC ACATGATATT CTAGATAATT
11881 CTCTCACAGG AATTAGAAAT GCCATAGCTG GAATGTTAGA TACGACAAAA TCACTAATTC
11941 GGGTTGGCAT AAATAGAGGA GGACTGACAT ATAGTTTGTG GAGGAAAATC AGTAATTACG
12001 ATCTAGTACA ATATGAAACA CTAAGTAGGA CTTTGCGACT AATTGTAAGT GATAAAATCA
12061 AGTATGAAGA TATGTGTTCC GTAGACCTTG CCATAGCATT GCGACAAAAG ATGTGGATTG
12121 ATTTATCAGG AGGAAGGATG ATAAGTGGAC TTGAAACGCC TGACCCATTA GAATTACTAT
12181 CTGGGGTAGT AATAACAGGA TCAGAACATT GTAAAATATG TTATCTTCA GATGGCACAA
12241 ACCCATATAC TTGGATGTAT TTACCCGGTA ATATCAAAAT AGGATCAGCA CATCTGCACA
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12361 TAGGATATAT CAAGAATCTT AGTAAACCTG CAAAAGCCGC AATAAGAATA GCAATGATAT
12421 ATACATGGGC ATTTGGTAAT GATGAGATAT CTTGGATGGA AGCCTCACAG ATAGCACAAA
12481 CACGTGCAAA TTTTACACTA GATAGTCTCA AAATTTTAAC ACCGGTAGCT ACATCAACAA
12541 ATTTATCACA CAGATTAAAG GATACTGCAA CTCAGATGAA ATTCTCCAGT ACATCATTGA
12601 TCAGAGTCAG CAGATTCATA ACAATGTCCA ATGATAACAT GTCTATCAAA GAAGCTAATG
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12781 TAGAAGATGA GTGTGTATT AAAGAAAGTT TTAATGATGA ACATATTAAT CCAGAGTCTA
12841 CATTAGAATT AATTCGATAT CCTGAAAGTA ATGAATTTAT TTATGATAAA GACCCACTCA
12901 AAGATGTGGA CTTATCAAAA CTTATGGTTA TTAAAGACCA TTCTTACACA ATTGATATGA
12961 ATTATTGGGA TGATACTGAC ATCATACATG CAATTTCAAT ATGTACTGCA ATTACAATAG
13021 CAGATACTAT GTCAACAATTA GATCGAGATA ATTTAAAAGA GATAATAGTT ATTGCAAATG
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13381 CCCTATCTAT ATGTGAATAT TCACTAGATC TATTTATGAG AGAATGGTTG AATGGTGTAT
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13621 ATATTAAGA AGACCCTACT CTTAAATATG TACAAATATC TGGATTATTA ATTAATCGT
13681 TCCCATCAAC TGTAACATAC GTAAGAAAGA CTGCAATCAA ATATCTAAGG ATTCGCGGTA
13741 TTAGTCCACC TGAGGTAATT GATGATTGGG ATCCGGTAGA AGATGAAAAT ATGCTGGATA
13801 ACATTGTCAA AACTATAAAT GATAACTGTA ATAAAGATAA TAAAGGGAAT AAAATTAACA
13861 ATTTCTGGGG ACTAGCACTT AAGAACTATC AAGTCCTTAA AATCAGATCT ATAACAAGTG
13921 ATTCTGATGA TAATGATAGA CTAGATGCTA ATACAAGTGG TTTGACACTT CCTCAAGGAG
13981 GGAATTATCT ATCGCATCAA TTGAGATTAT TCGGAATCAA CAGCACTAGT TGTCTGAAAG
14041 CTCTTGAGTT ATCACAATTT TTAATGAAGG AAGTCAATAA AGACAAGGAC AGGCTCTTCC
14101 TGGGAGAAGG AGCAGGAGCT ATGCTAGCAT GTTATGATGC CACATTAGGA CCTGCAGTTA
14161 ATTTATATAA TTCAGGTTTG AATATAACAG ATGTAATTGG TCAACGAGAA TTGAAAATAT
14221 TTCCTTCAGA GGTATCATTG GTAGGTAAAA AATTAGGAAA TGTGACACAG ATTCTTAACA
14281 GGGTAAAAGT ACTGTTCAAT GGGAATCCTA ATTCAACATG GATAGGAAAT ATGGAATGTG
14341 AGAGCTTAAT ATGGAGTGAA TTAAATGATA AGTCCATTGG ATTAGTACAT TGTGATATGG
14401 AAGGAGCTAT CGGTAAATCA GAAGAACTG TTCTACATGA ACATTATAGT GTTATAAGAA
14461 TTACATACTT GATTGGGGAT GATGATGTTG TTTTAGTTTC CAAAATTATA CCTACAATCA

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In a third strategy (Figure 19), chimeric PIV3-PIV2 F and HN genes were constructed in which regions of the PIV2 F and HN ORFs encoding the ectodomains and the transmembrane domains were amplified from pLit.PIV32Fhc and pLit.PIV32HNhc, respectively, using PCR, Vent DNA polymerase, and primer pairs specific to PIV2 F (13, 14 in Table 22) and PIV2 HN (15, 16 in Table 22). In parallel, the partial ORFs of PIV3 F and HN genes encoding the ectodomains plus transmembrane domains were deleted from their cDNA subclones pLit.PIV3.F3a and pLit.PIV3.HN4 (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), respectively, using PCR, Vent DNA polymerase, and primer pairs specific to PIV3 F (17, 18 in Table 22) and PIV3 HN (19, 20 in Table 22). The F and HN cDNA fragments of PIV2 and PIV3 were gel purified and ligated to generate pLit.PIV32FCT and pLit.PIV32HNCT, respectively. The chimeric F and HN constructs were digested with PpuMI plus SpeI and assembled together to generate pLit.PIV32CT, which was sequenced across the PIV specific region in its entirety and found to be as designed. The 4 kb BspEI-SpeI fragment from pLit.PIV32CT was introduced into the BspEI-SpeI window of p38'ΔPIV31hc to generate p38'ΔPIV32CT. The 6.5 kb BspEI-SphI fragment from p38'ΔPIV32CT, containing the PIV3-PIV2 F and HN chimeric genes, was introduced into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45, to generate pFLC.PIV32CT (Table 25, SEQ ID NO. 62) and pFLC.PIV32CTcp45, respectively. The nucleotide sequence of this *BspEI-SpeI* fragment is submitted in the GenBank.

TABLE 25 (SEQ ID NO. 62)

Sequence of pFLC.PIV32CT, 15474 bp in sense orientation
(only the insert is shown)

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1  ACCAAACAAG AGAAGAAACT TGTCTGGGAA TATAAATTTA ACTTTAAATT AACTTAGGAT
61  TAAAGACATT GACTAGAAGG TCAAGAAAAG GGAACCTCTAT AATTTCAAAA ATGTTGAGCC
121 TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA
181 TCATTCCTGG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA ATAAGTATG
241 ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT AATGAGAAAC
301 AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG
361 AGCTCTACCT AACAACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA TACATGATTG
421 AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAAACTATGT
541 TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT
601 CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTCAGACAA GATGGAACAG
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781 CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAATGAAT ACCAGCAGAA
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15001	AGGGAAAGTT	AAGACTGCTA	TCGAGAAGAC	TAGTATTAAG	TTGGATTTCA	TTATCATTAT
15061	CGACTCGATT	ACTTACAGGT	CGCTTTCCTG	ATGAAAAATT	TGAACATAGA	GCACAGACTG
15121	GATATGTATC	ATTAGCTGAT	ACTGATTTAG	AATCATTAAA	GTTATTGTCT	AAAAACATCA
15181	TTAAGAATTA	CAGAGAGTGT	ATAGGATCAA	TATCATATTG	GTTTCTAACC	AAAGAAGTTA
15241	AAATACTTAT	GAAATTGATC	GGTGGTGCTA	AATTATTAGG	AATTCCCAGA	CAATATAAAG
15301	AACCCGAAGA	CCAGTTTATTA	GAACAATACA	ATCAACATGA	TGAATTTGAT	ATCGATTAAA
15361	ACATAAAATAC	AATGAAGATA	TATCCTAACC	TTTATCTTTA	AGCCTAGGAA	TAGACAAAAA
15421	GTAAGAAAAA	CATGTAATAT	ATATATACCA	AACAGAGTTC	TTCTCTTGTT	TGGT

The cDNA engineering was designed so that the final PIV3-2 antigenomes conformed to the rule of six (Calain et al., *J. Virol.* 67:4822-30, 1993; Durbin et al., *Virology* 234:74-83, 1997, each incorporated herein by reference). The PIV3-2 insert in pFLC.PIV32TM is 15498 nt in length, and that in pFLC.PIV32CT is 15474 nt in length.

- 5 These total lengths do not include two 5'-terminal G residues contributed by the T7 promoter, because it is assumed that they are removed during recovery.

Transfection and recovery of recombinant chimeric PIV3-PIV2 viruses

- Hep-2 cell monolayers were grown to confluence in six-well plates, and transfections were performed essentially as described (Tao et al., 72:2955-2961, 1998, incorporated herein by reference). The Hep-2 monolayer in one well was transfected with 5 µg PIV3-PIV2 antigenomic cDNA and three support plasmids, 0.2 µg pTM(N), 0.2 µg pTM(PnoC), 0.1 µg pTM(L) in 0.2 ml of MEM containing 12 µl LipofectACE (Life Technologies). The cells were infected simultaneously with MVA-T7 at a multiplicity of infection (MOI) of 3 in 0.8 ml of serum-free MEM containing 50 µg/ml gentamicin and 2 mM glutamine. The chimeric antigenomic cDNA pFLC.2G+.hc (Tao et al., *J. Virol.* 72:2955-2961, 1998), was transfected in parallel as a positive control. After incubation at 32°C for 12 hours, the transfection medium was replaced with 1.5 ml of fresh serum-free MEM supplemented with 50 µg/ml gentamicin and 2 mM glutamine. Transfected cells were incubated at 32°C for two additional days. Gamma-irradiated porcine trypsin (p-trypsin; T1311, Sigma, St Louis, MO) was added to a final concentration of 0.5 µg/ml on day 3 post transfection. Cell culture supernatants were harvested and passaged (referred to as passage 1) onto fresh Vero cell monolayers in T25 flasks. After overnight adsorption, the transfection harvest was replaced with fresh VP-SFM supplemented with 0.5 µg/ml p-trypsin. Cultures from passage 1 were incubated at 32°C for 4 days, and the amplified virus was harvested and further passaged on Vero cells (referred to as passage 2) for another 4 days at 32°C in the presence of 0.5 µg/ml p-trypsin. The presence of viruses in the passage 2 cultures was determined by hemadsorption with 0.2% guinea pig red blood cells (RBCs). Viruses were further purified by three consecutive terminal dilutions performed using Vero cells maintained in VP-SFM supplemented with 2 mM glutamine, 50 µg/ml gentamicin, and 0.5 µg/ml p-trypsin. Following the third terminal dilution, virus was further amplified three times on Vero cells, and this virus suspension was used for further characterization *in vitro* and *in vivo*.

Confirmation of the chimeric nature of vRNA using sequencing and restriction analysis of PCR products

For analysis of the genetic structure of vRNAs, the recombinant PIVs were amplified on LLC-MK2 cells and concentrated. vRNA was extracted from the viral pellets and reverse transcribed using the Superscript Preamplification System. RT-PCR was performed using the Advantage cDNA synthesis kit and primer pairs specific to PIV2 or PIV3 (21, 22 or 23, 24 in Table 22). RT-PCR products were either analyzed by restriction digestion or gel purified and analyzed by sequencing.

Replication of PIVs in LLC-MK2 cells

Growth of the PIV viruses in tissue culture was evaluated by infecting confluent LLC-MK2 cell monolayers on six-well plates in triplicate at an MOI of 0.01. The inoculum was removed after absorption for 1 hour at 32°C. Cells were washed 3 times with serum-free OptiMEM I, fed with 2 ml/well of OptiMEM I supplemented with 50 µg/ml gentamicin and 0.5 µg/ml p-trypsin, and incubated at 32°C. At each 24 hour interval, a 0.5 ml aliquot of medium was removed from each well and flash-frozen, and 0.5 ml fresh medium with p-trypsin was added to the cultures. The virus in the aliquots was titrated at 32°C on LLC-MK2 cell monolayers using fluid overlay as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), and the endpoint of the titration was determined by hemadsorption, and the titers are expressed as log₁₀TCID₅₀/ml.

Replication of recombinant chimeric PIV3-PIV2 viruses at various temperatures

Viruses were serially diluted in 1X L15 supplemented with 2 mM glutamine and 0.5 µg/ml p-trypsin. Diluted viruses were used to infect LLC-MK2 monolayers in 96 well plates. Infected plates were incubated at various temperatures for 7 days as described (Skiadopoulos et al., *Vaccine* 18:503-510, 1999, incorporated herein by reference). Virus titers were determined as above.

Replication, immunogenicity, and protective efficacy of recombinant chimeric PIV3-PIV2 viruses in the respiratory tract of hamsters

Golden Syrian hamsters in groups of six were inoculated intranasally with 10^{5.3} TCID₅₀ of recombinant or biologically-derived viruses. Four days after inoculation, hamsters were sacrificed and their lungs and nasal turbinates were harvested and prepared

for quantitation of virus (Skiadopoulos et al., Vaccine 18:503-510, 1999, incorporated herein by reference). The titers are expressed as mean \log_{10} TCID₅₀/gram of tissue for each group of six hamsters.

Hamsters in groups of 12 were infected intranasally with $10^{5.3}$ TCID₅₀ of viruses on day 0, and six hamsters from each group were challenged four weeks later with 10^6 TCID₅₀ of PIV1 or 10^6 TCID₅₀ of PIV2. Hamsters were sacrificed 4 days after challenge and their lungs and nasal turbinates were harvested. Challenge virus titers in the harvested tissue was determined as previously described (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference). The virus titers are expressed as mean \log_{10} TCID₅₀/gram of tissue for each group of six hamsters. Serum samples were collected three days prior to inoculation and on day 28, and hemagglutination-inhibition antibody (HAI) titers against PIV1, PIV2, and PIV3 were determined as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985, incorporated herein by reference). The titers are expressed as reciprocal mean \log_2 .

Replication, immunogenicity, and protective efficacy of recombinant chimeric PIV3-PIV2 viruses in African green monkeys (AGMs)

AGMs in groups of 4 were infected intranasally and intratracheally with 10^5 TCID₅₀ of virus at each site on day 0. Nasal/throat (NT) swab specimens and tracheal lavages were collected for 12 and 5 days, respectively, as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985). On day 29, immunized AGMs were challenged intranasally and intratracheally with 10^5 TCID₅₀ of PIV2/V94 at each site. NT swab specimens and tracheal lavages were collected for 10 and 5 days, respectively. Pre-immunization, post-immunization, and post challenge serum samples were collected on days -3, 28, and 60, respectively. Virus titers in the NT swab specimens and in tracheal lavages were determined as previously described (Tao et al., J. Virol. 72:2955-2961, 1998). Titers are expressed as \log_{10} TCID₅₀/ml. Serum neutralizing antibody titers against PIV1 and PIV2 were determined as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985), and the titers are expressed as reciprocal mean \log_2 .

Replication and immunogenicity of recombinant chimeric PIV3-PIV2 viruses in chimpanzees

Chimpanzees in groups of 4 were infected intranasally and intratracheally with 10^5 TCID₅₀ of PIV2/V94 or rPIV3-2TM on day 0 as previously described (Whitehead et al., *J. Virol.* 72:4467-4471, 1998, incorporated herein by reference). NT swab specimens were collected daily for 12 days and tracheal lavages were obtained on days 2, 4, 6, 8, and 10. Virus titers in the specimens were determined as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference). The peak virus titers are expressed as mean log₁₀TCID₅₀/ml. Pre-immunization and post-immunization serum samples were collected on days -3 and 28, respectively. Serum neutralizing antibody titers against PIV1 and PIV2 were determined as previously described (van Wyke Coelingh et al., *Virology* 143:569-582, 1985, incorporated herein by reference), and the titers are expressed as reciprocal mean log₂.

Viable recombinant chimeric virus was not recovered from PIV3-PIV2 chimeric cDNA encoding the complete PIV2 F and HN proteins

The construction of the PIV3-PIV2 chimeric cDNA, in which the F and HN ORFs of the JS wild type PIV3 were replaced by those of PIV2/V94, is described above and summarized in Figure 17. The final plasmid construct, pFLC.PIV32hc (Figure 17), encodes a PIV3-PIV2 chimeric antigenomic RNA of 15492 nt, which conforms to the rule of six.

HEp-2 cell monolayers were transfected with pFLC.PIV32hc along with the three support plasmids pTM(N), pTM(PnoC), and pTM(L) using LipofectACE, and the cells were simultaneously infected with MVA-T7 as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference). Virus was not recovered from several initial transfections using pFLC.PIV32hc, while chimeric viruses were recovered from all the transfections using control plasmid pFLC.2G+.hc.

Consistent with these results is the possibility that a mutation occurred outside of the 4 kb BspEI-SpeI segment of pFLC.PIV32hc that prevented the recovery of rPIV3-2 virus from cells transfected with this cDNA clone. To examine this possibility, the BspEI-SpeI fragments of p38'ΔPIV31hc and p38'ΔPIV32hc were exchanged. The regenerated p38'ΔPIV31hc and p38'ΔPIV32hc were identical to those in Figure 17 except that the SpeI-SphI fragments containing PIV3 L gene sequences were exchanged. The BspEI-SphI fragments of these two regenerated cDNAs were introduced into the BspEI-SphI window of a PIV3 full-length clone, p3/7-(131)2G+, in five separate independent ligations to give 10 pFLC.2G+.hc and pFLC.PIV32hc clones (2 clones selected from each ligation),

Genetic characterization of recombinant chimeric PIV3-PIV2 viruses

The biologically-cloned chimeric PIV3-PIV2 viruses, rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, and rPIV3-2CTcp45, were propagated on LLC-MK2 cells and then concentrated. Viral RNAs extracted from pelleted viruses were used in RT-PCR amplification of specific gene segments using primer pairs specific to PIV2 or PIV3 (21, 22 or 23, 24 in Table 22). The restriction enzyme digestion patterns of the RT-PCR products amplified with PIV2 specific primer pairs from rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, and rPIV3-2CTcp45, were each distinct from that derived from PIV2/V94, and their patterns, using EcoRI, MfeI, NcoI, or PpuMI, were those expected from the designed cDNA. Nucleotide sequences for the 8 different PIV3-PIV2 junctions in F and HN genes of rPIV3-2TM and rPIV3-2CT are given in Figure 20. Also, the *cp45* markers present in rPIV3-2TMcp45 and rPIV3-2CTcp45, except those in the 3'-leader region and the gene start of NP, were verified with RT-PCR and restriction enzyme digestion as previously described (Skiadopoulos et al., *J Virol.* 73:1374-81, 1999, incorporated herein by reference). These results confirmed the chimeric nature of the recovered PIV3-PIV2 viruses as well as the presence of the introduced *cp45* mutations.

PIV3-PIV2 recombinant chimeric viruses replicate efficiently in LLC-MK2 cells *in vitro*

The kinetics and magnitude of replication *in vitro* of the PIV3-PIV2 recombinant chimeric viruses were assessed by multicycle replication in LLC-MK2 cells (Figure 21). LLC-MK2 cell monolayer cultures in six-well plates were infected in triplicate with rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, or rPIV3-2CTcp45 at an MOI of 0.01 in the presence of p-trypsin (0.5 µg/ml). Samples were removed from culture supernate at 24 hour intervals for 6 days. Each of the recombinant chimeric viruses, except rPIV3-2CTcp45 (clone 2A1), replicated at the same rate and to a similar level as their PIV2/V94 parent virus indicating that PIV3-PIV2 chimerization of F and HN proteins did not alter the rates of growth of the recombinant chimeric viruses, and all reached a titer of 10^7 TCID₅₀/ml or higher. Only the rPIV3-2CTcp45 grew slightly faster in each of two experiments and reached its peak titer earlier than PIV2/V94. This accelerated growth pattern was likely a result of an unidentified mutation in this clone since a sister clone failed to exhibit this growth pattern. rPIV3-2CTcp45 clone 2A1 was used in the studies described below.

The level of temperature sensitivity of rPIV3-2 chimeric viruses and their *cp45* derivatives

The level of temperature sensitivity of replication of PIV3-PIV2 recombinant chimeric viruses was tested to determine if rPIV3-2TM and rPIV3-2CT viruses exhibit a *ts* phenotype and to determine if the acquisition of the 12 *cp45* mutations by these viruses specified a level of temperature sensitivity characteristic of *cp45* derivatives bearing these 12 PIV3 *cp45* mutations (Skiadopoulos et al., J Virol. 73:1374-81, 1999, incorporated herein by reference). The level of temperature sensitivity of the virus was determined in LLC-MK2 cell monolayers as previously described (Skiadopoulos et al., Vaccine 18:503-510, 1999, incorporated herein by reference) (Table 26). The titer of rPIV3-2TM and rPIV3-2CT was fairly constant at permissive temperature (32°C) and the various restrictive temperatures tested indicating these recombinants were *ts*+. In contrast, their *cp45* derivatives, rPIV3-2TMcp45 and rPIV3-2CTcp45, were *ts* and the level of temperature sensitivity was similar to that of rPIV3-1cp45, the chimeric PIV3-PIV1 virus carrying the complete PIV1 F and HN glycoproteins and the same set of 12 *cp45* mutations. Thus the *in vitro* properties of rPIV3-2TM and rPIV3-2CT viruses and their *cp45* derivative are similar to those of rPIV3-1 and rPIV3-1cp45, respectively, suggesting that the *in vivo* properties of the rPIV3-2 and rPIV3-1 viruses would also be similar, but surprisingly this was not the case.

Table 26. The replication of rPIV3-2CT and rPIV3-2TM are not temperature sensitive in LLC-MK2 cells, whereas the inclusion of the *cp45* mutations confers the *cp45* temperature sensitive phenotype

Virus	Titer at 32° C ^a (log ₁₀ TCID ₅₀)	Change in titer (log ₁₀) at various temperatures compared to that at 32° ^{a, b}					
		35° ^c	36°	37°	38°	39°	40°
rPIV3/JS	7.9	0.3 ^b	0.1	0.1	(0.3) ^b	(0.4)	0.4
PIV3 _{cp45} ^e	7.8	0.5	0.3	1.3	<u>3.4</u> ^d	6.8	6.9
PIV1/Wash64 ^e	8.5	1.5	1.1	1.4	0.6	0.5	0.9
rPIV3-1	8.0	0.8	0.5	0.6	0.9	1.1	2.6
rPIV3-1 _{cp45}	8.0	0.5	0.4	<u>3.4</u> ^d	4.8	6.6	7.5
PIV2/V9412 ^e	7.8	0.3	(0.1)	0.0	(0.4)	(0.4)	0.0
rPIV3-2CT	6.9	0.3	0.3	0.6	(0.1)	0.6	0.4
rPIV3-2TM	8.3	0.3	(0.1)	0.3	0.6	1.0	<u>2.1</u> ^d
rPIV3-2CT _{cp45}	8.0	0.8	(0.4)	<u>2.0</u> ^d	4.3	7.5	≥7.6
rPIV3-2TM _{cp45}	8.0	0.3	0.6	<u>2.4</u> ^d	5.4	7.5	≥7.6

^a Data presented are means of two experiments.

^b Numbers not in parentheses represent titer decrease; numbers in parentheses represent titer increase.

^c Data at 35° were from one experiment only.

^d Values which are underlined represent the lowest temperature at which there was a 100-fold reduction of virus titer compared to the titer at permissive temperature (32°C). This restrictive temperature is referred to as the shut-off temperature.

^e Biologically-derived viruses.

rPIV3-2TM and rPIV3-2CT are attenuated, immunogenic, and highly protective in hamsters, and introduction of *cp45* mutations results in highly attenuated and less protective viruses

Hamsters in groups of six were inoculated intranasally with $10^{5.3}$ TCID₅₀ of rPIV3-2TM, rPIV3-2CT, rPIV3-2TM*cp45*, rPIV3-2CT*cp45*, or control viruses. It was previously seen that rPIV3-1 virus replicated in the upper and lower respiratory tract of hamsters like that of its PIV3 and PIV1 parents (Skiadopoulos et al., Vaccine 18:503-510, 1999; Tao et al., J. Virol. 72:2955-2961, 1998, each incorporated herein by reference). PIV2 virus replicates efficiently in hamsters, but rPIV3-2TM and rPIV3-2CT viruses each replicated to a 50- to 100-fold lower titer than their PIV2 and PIV3 parents in the upper respiratory tract and to a 320- to 2000-fold lower titer in the lower respiratory tract (Table 27). This indicates that the chimeric PIV3-PIV2 F and HN glycoproteins specify an unexpected attenuation phenotype in hamsters. rPIV3-2TM*cp45* and rPIV3-2CT*cp45*, the derivatives carrying the *cp45* mutations, were 50- to 100-fold more attenuated than their respective rPIV3-2 parents, with only barely detectable replication in the nasal turbinates, and none in the lungs. These rPIV3-2*cp45* viruses were clearly more attenuated than rPIV3-1*cp45*, exhibiting an additional 50-fold reduction of replication in the nasal turbinates. Thus, the attenuating effects of the chimerization of F and HN glycoproteins and that specified by *cp45* mutations were additive.

Table 27. The rPIV3-2TM and rPIV3-2CT viruses, in contrast to rPIV3-1, are attenuated in the respiratory tract of hamsters and importation of the *cp45* mutations resulted in further attenuation.

Group] ^c Virus ^a	Virus titers in the indicated tissue (log ₁₀ TCID ₅₀ /g ± S.E.) ^b [Duncan			
	NT	log ₁₀ titer reduction	Lung	log ₁₀ titer reduction
rPIV3/JS	5.9 ± 0.1[AB]	0	6.5 ± 0.1[A]	0
rPIV3 <i>cp45</i>	4.5 ± 0.2[C]	1.4 ^c	1.8 ± 0.2[E]	4.7 ^c
PIV1/Wash64 ^d	5.7 ± 0.1[B]	-	5.5 ± 0.1[B]	-
rPIV3-1	6.4 ± 0.2[A]	0	6.6 ± 0.2[A]	0
rPIV3-1 <i>cp45</i>	3.1 ± 0.1[D]	3.3 ^c	1.2 ± 0.0[F]	5.4 ^c
PIV2/V94 ^d	6.2 ± 0.2[A]	0	6.4 ± 0.2[A]	0
rPIV3-2CT	4.5 ± 0.4[C]	1.7 ^c	3.1 ± 0.1[D]	3.3 ^c
rPIV3-2TM	3.9 ± 0.3[C]	2.3 ^c	3.9 ± 0.4[C]	2.5 ^c
rPIV3-2CT <i>cp45</i>	1.4 ± 0.1[E]	4.8 ^c	1.5 ± 0.2[E]	4.9 ^c
rPIV3-2TM <i>cp45</i>	1.6 ± 0.2[E]	4.6 ^c	1.4 ± 0.1[E]	5.0 ^c

^a Hamsters in group of six were inoculated intranasally with 10^{5.5} TCID₅₀ of indicated virus on day 0.

^b Hamsters were sacrificed and their tissue samples harvested on day 4. The virus titer in hamster tissues was determined and the results are expressed as log₁₀TCID₅₀/g ± standard error (SE). NT = nasal turbinates.

^c The log₁₀ titer reduction values are derived by comparing: rPIV3*cp45* against rPIV3/JS; rPIV3-1*cp45* against rPIV3-1; each of the PIV3-PIV2 chimeras against PIV2/V94.

^d Biologically-derived viruses.

^e Grouping as analyzed by Duncan mult:range test.

Table 28. The rPIV3-2CT and rPIV3-2TM viruses are highly protective in hamsters against challenge with wild type PIV2, but not against PIV1

Immunizing virus ^a	HAI antibody titer ^b against indicated virus (reciprocal mean log ₂ ± SE)				Challenge virus titer ^c in indicated tissue (log ₁₀ TCID ₅₀ /g ± SE)			
					PIV1		PIV2	
	PIV1	PIV2	PIV3		NT	Lung	NT	Lung
rPIV3/JS	≤1	≤1	10.2±0.1		6.2 ± 0.2	5.8 ± 0.1	5.9±0.2	5.7±0.2
rPIV3 _{cp45}	≤1	≤1	8.6±0.2		5.9 ± 0.3	5.1 ± 0.3	5.6±0.2	4.5±0.7
PIV1	6.7±0.2	≤1	≤1		1.3 ± 0.1	≤1.2 ± 0.0	6.1±0.2	6.2±0.3
rPIV3-1	6.4±0.2	≤1	≤1		≤1.2 ± 0.0	≤1.2 ± 0.0	6.5±0.2	5.0±0.6
rPIV3-1 _{cp45}	1.8±0.6	≤1	≤1		3.9 ± 0.4	1.6 ± 0.3	6.2±0.2	4.5±0.6
PIV2	≤1	4.0±0.0	≤1		5.9 ± 0.2	5.5 ± 0.1	≤1.2±0.0	≤1.2±0.0
rPIV3-2CT	≤1	3.6±0.8	≤1		5.3 ± 0.1	5.2 ± 0.3	≤1.2±0.0	≤1.2±0.0
rPIV3-2TM	≤1	4.5±0.2	≤1		5.9 ± 0.2	4.4 ± 0.3	≤1.2±0.0	≤1.2±0.0
rPIV3-2CT. _{cp45}	≤1	≤1	≤1		6.2 ± 0.2	5.7 ± 0.1	5.3±0.2	3.3±0.8
rPIV3-2TM. _{cp45}	≤1	≤1	≤1		5.8 ± 0.3	4.4 ± 0.3	5.5±0.2	3.7±0.7

^a Hamsters in groups of 12 were immunized intranasally with 10^{5.3} TCID₅₀ of the indicated virus on day 0.

^b Serum samples were collected two days before immunization and 28 days after immunization. They were tested for HAI antibody titer against the three PIVs, and the antibody titers are presented as reciprocal mean log₂ ± standard error (SE).

^c Six hamsters from each group were challenged intranasally with 10⁶ TCID₅₀ of PIV1 (on day 29) or PIV2 (on day 32). Hamster tissues were harvested 4 days after challenge, and the virus titer in indicated tissues are expressed as log₁₀ TCID₅₀/g ± SE.

rPIV3-2TM and rPIV3-2CT are attenuated, immunogenic, and highly protective in AGMs, whereas introduction of *cp45* mutations results in highly attenuated and poorly protective viruses

Certain recombinant PIV3 and RSV viruses may exhibit different levels of attenuation in rodents and primates (Skiadopoulos et al., Vaccine 18:503-510, 1999; Skiadopoulos et al., J. Virol. 73:1374-81, 1999a; Skiadopoulos et al., Virology 272:225-34, 2000; Whitehead et al., J Virol. 73:9773-9780, 1999, each incorporated herein by reference), indicating that attenuation can be somewhat species specific. Therefore, the rPIV3-2 viruses were evaluated for their level of replication and immunogenicity in AGMs. AGMs in groups of four were intranasally and intratracheally administered 10^5 TCID₅₀ per site of rPIV3-2TM, rPIV3-2CT, rPIV3-2TM*cp45*, rPIV3-2CT*cp45*, PIV2/V94, or rPIV3-1 on day 0. Virus in the NT swab specimens (collected day 1 to 12) and tracheal lavages (collected on day 2, 4, 5, 8, and 10) were titrated as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985, incorporated herein by reference). As shown in Table 29, rPIV3-2TM and rPIV3-2CT were clearly attenuated in the respiratory tract of AGMs as indicated by a peak titer of virus shedding lower in both the upper and lower respiratory tract than their PIV2/V94 parent virus.

rPIV3-2TM*cp45* and rPIV3-2CT*cp45*, the derivatives carrying *cp45* mutations, were detected at very low levels, if at all, in the NT swab and tracheal lavage specimens suggesting that the attenuating effects of chimerization of the F and HN glycoproteins and that specified by the *cp45* mutations were additive for AGMs as well as for hamsters.

To determine whether immunization of AGMs with the PIV3-PIV2 chimeric viruses is protective against PIV2 challenge, AGMs previously infected with a rPIV3-2 virus were challenged with 10^5 TCID₅₀ of PIV2 on day 28 (Table 29). Virus present in the NT swab specimens (collected day 29 to 38) and tracheal lavages fluids (collected on day 30, 32, 34, 36, and 38) was titrated as previously described (Durbin et al., Virology 261:319-30, 1999, incorporated herein by reference). As shown in Table 29, immunization with rPIV3-2TM and rPIV3-2CT induced a high level of restriction of the replication of PIV2/V94 challenge virus. In contrast, immunization of AGMs with rPIV3-2TM*cp45* and rPIV3-2CT*cp45* failed to restrict the replication of PIV2/V94 challenge virus and these animals developed very low levels of pre-challenge serum neutralizing antibody to PIV2. The

complete restriction of replication of PIV2/V94 challenge virus in rPIV3-2CT immunized AGMs was associated with a 2.5-fold greater level of pre-challenge serum antibody to PIV2 than that of rPIV3-2TM immunized AGMs which provided incomplete protection.

Table 29. The rPIV3-2CT or rPIV3-2TM viruses are attenuated for replication in the respiratory tract of African green monkeys, yet still induce resistance to challenge with wild type PIV2

Immunizing ^a virus	Mean peak titer ^b of immunizing virus in indicated site (log ₁₀ TCID ₅₀ /ml ± SE)		Serum neutralization antibody titer ^c against indicated virus (mean reciprocal log ₂ ± SE)		Mean peak titer ^d of PIV2/V94 challenge virus in indicated site (log ₁₀ TCID ₅₀ /ml ± SE)	
	NT	TL	PIV1	PIV2	NT	TL
rPIV3-1	2.6±0.5	3.2±0.1	6.3±0.4	3.1±0.3	3.6±0.2	3.3±0.7
PIV2/V94	2.8±0.7	5.0±0.3	3.8±0.0	7.1±0.7	≤0.2	≤0.2
rPIV3-2CT	1.5±0.4	0.5±0.2	2.9±0.1	7.2±0.1	≤0.2	≤0.2
rPIV3-2TM	1.4±0.1	1.6±0.7	4.1±0.1	5.9±0.2	1.6±0.6	1.3±0.9
rPIV3-2CT _{cp45}	1.0±0.2	≤0.2	4.1±0.1	5.3±0.0	3.3±0.4	3.5±0.3
rPIV3-2TM _{cp45}	0.6±0.3	≤0.2	3.4±0.2	4.6±0.6	3.0±0.5	4.1±0.2

^a African green monkeys in group of 4 were inoculated with 10⁵ TCID₅₀ of indicated virus intranasally and intratracheally on day 0.

^b Combined nasal wash and throat swab (NT) samples were collected on days 1 to 12. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10. The virus titers were determined on LLC-MK2 monolayers and expressed as log₁₀TCID₅₀/ml ± standard error (SE).

^c Serum samples collected on day 28 were assayed for their neutralizing antibody titers against PIV1 and PIV2. The titers were expressed as reciprocal mean log₂ ± SE.

^d NT specimens were collected on days 29 to 38. TL specimens were collected on days 30, 32, 34, 36, and 38.

rPIV3-2TM is attenuated in its replication in the respiratory tract of chimpanzees

Chimpanzees in groups of 4 were inoculated intranasally and intratracheally with 10^5 TCID₅₀ of rPIV3-2TM or PIV2/V94 on day 0. NT swab specimens (day 1 to 12) and tracheal lavage (days 2, 4, 6, 8, and 10) samples were collected. Virus titer was determined as previously described (Durbin et al., Virology 261:319-30, 1999, incorporated herein by reference), and results are expressed as log₁₀TCID₅₀/ml. As shown in Table 30, rPIV3-2TM had a lower peak titer than its wild type parent PIV2/V94 and was shed for a significantly shorter duration than PIV2/V94, indicating that rPIV3-2TM is attenuated in chimpanzees. PIV2/V94 wt virus replicates to low levels in chimpanzees compared to hamsters and AFGs, whereas rPIV3-2TM virus was attenuated in each of these model hosts.

As noted above, the major protective antigens of PIVs are their HN and F glycoproteins. Thus, in exemplary embodiments of the invention, live attenuated PIV candidate vaccine viruses for use in infants and young children include chimeric HPIV3-1 and HPIV3-2 viruses carrying full-length PIV1 and partial PIV2 glycoproteins, respectively
 5 in a PIV3 background genome or antigenome. In the latter case, chimeric HN and F ORFs rather than full-length PIV2 ORFs are used to construct the full-length cDNA. The recovered viruses, designated rPIV3-2CT in which the PIV2 ectodomain and transmembrane domain is fused to the PIV3 cytoplasmic domain and rPIV3-2TM in which the PIV2
 10 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail domain, possessed similar *in vitro* and *in vivo* phenotypes. In particular, the rPIV3-2 recombinant chimeric viruses exhibit a host range phenotype, i.e. they replicate efficiently *in vitro* but are restricted in replication *in vivo*. This attenuation *in vivo* occurs in the absence of any added mutations from *cp45*. This is an unexpected host range effect which is highly desirable for vaccine
 15 purposes, in part because the phenotype is not specified by point mutations which may refer to wt. At the same time, the unrestricted growth *in vitro* is highly advantageous for efficient vaccine production.

Although rPIV3-2CT and rPIV3-2TM replicate efficiently *in vitro*, they are highly attenuated in both the upper and the lower respiratory tract of hamsters and African green monkeys (AGMs), indicating that chimerization of the HN and F proteins of PIV2 and
 20 PIV3 itself specified an attenuation phenotype *in vivo*. Despite this attenuation, they are highly immunogenic and protective against challenge with PIV2 wild virus in both species. rPIV3-2CT and rPIV3-2TM were further modified by the introduction of the 12 PIV3 *cp45* mutations located outside of the HN and F coding sequences to derive rPIV3-2CT*cp45* and rPIV3-2TM*cp45* which replicated efficiently *in vitro* but were even further attenuated in
 25 hamsters and AGMs indicating that the attenuation specified by the glycoprotein chimerization and by the *cp45* mutations was additive.

The development of antigenic chimeric viruses possessing protective antigens of one virus and attenuating mutations from another virus has been reported by others for influenza viruses (Belshe et al., N. Engl. J. Med. 338:1405-1, 1998; Murphy et al., Infectious
 30 Diseases in Clinical Practice 2:174-181, 1993) and for rotaviruses (Perez-Schael et al., N. Engl. J. Med. 337:1181-7, 1997). Attenuated antigenic chimeric vaccines are more readily generated for these viruses which have segmented genomes, since genome segment

reassortment occurs with high frequency during coinfection. Live attenuated influenza virus vaccine candidates are antigenically updated annually by replacement of the HA and NA genes of the attenuated donor virus with those of a new epidemic or pandemic virus.

Antigenic chimeric viruses have also been produced for single-stranded, negative-sense RNA viruses. For example, antigenic chimeric PIV1 vaccine candidates can be constructed according to the methods disclosed herein by substituting the full-length HN and F proteins of parainfluenza virus type 1 for those of PIV3 in an attenuated PIV3 vaccine candidate, and this recombinant is attenuated and protective against PIV1 challenge in experimental animals. Similarly, exemplary antigenic chimeric respiratory syncytial virus (RSV) vaccine candidates can be made in which one or more of the RSV F and G protective antigens, or antigenic determinant(s) thereof, of subgroup B virus are substituted for those in an attenuated RSV subgroup A virus yielding attenuated RSV subgroup B vaccine candidates. (See also, International Publication No. WO 97/06270; Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567 (1995); U.S. Patent Application No. 08/892,403, filed

In the present disclosure, viable recombinant virus in which the full-length PIV2 HN or F protein was used to replace those of PIV3 was not recovered in this instance, evidently attributable to incidental mutations introduced during cDNA construction, whereas this was successfully achieved for the PIV1-PIV3 glycoprotein exchange. This suggests that the PIV2 HN or F glycoprotein is poorly compatible with one or more of the PIV3 proteins encoded in the cDNA. Two viable PIV2-PIV3 chimeric viruses were obtained when chimeric HN and F ORFs rather than full-length PIV2 ORF were used to construct the full-length cDNA. One of these chimeric viruses contained chimeric HN and F glycoproteins in which the PIV2 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail region, and the other contained chimeric HN and F glycoproteins in which the PIV2 ectodomain and transmembrane region was fused to the PIV3 cytoplasmic tail region. Both rPIV3-2 recombinants possessed similar, although not identical, *in vitro* and *in vivo* phenotypes. Thus, it appeared that only the cytoplasmic tail of the HN or F glycoprotein of PIV3 was required for successful recovery of the PIV2-PIV3 chimeric viruses.

In previous studies directed to protein structure-function analysis, chimeric HN or F proteins have been constructed and expressed *in vitro* and have been used to map various functional domains of the proteins (Bousse et al., Virology 204:506-14, 1994; Deng et al., Arch. Virol. Suppl. 13:115-30, 1997; Deng, et al., Virology 253:43-54, 1999; Deng et

al., *Virology* 209:457-69, 1995; Mebatsion et al., *J. Virol.* 69:1444-1451, 1995; Takimoto et al., *J. Virol.* 72:9747-54, 1998; Tanabayashi et al., *J. Virol.* 70:6112-6118, 1996; Tsurudome et al., *J. Gen. Virol.* 79:279-89, 1998; Tsurudome et al., *Virology* 213:190-203, 1995; Yao et al., *J. Virol.* 69:7045-53, 1995). In one report, a chimeric glycoprotein consisting of a
5 measles virus F cytoplasmic tail fused to the transmembrane and ectodomains of the vesicular stomatitis virus G protein was inserted into a measles virus infectious clone in place of the measles virus F and HN virus glycoproteins (Spielhofer et al., *J. Virol.* 72:2150-9, 1998). A chimeric virus was obtained that was replication competent, but highly restricted in replication *in vitro* as indicated by delayed growth and by low virus yields
10 indicating a high degree of attenuation *in vitro*. This finding is in marked contrast to the phenotype exhibited by recombinant PIV of the invention expressing chimeric glycoproteins, e.g., a PIV2-PIV3 chimera, which replicate efficiently *in vitro*.

The efficient replication of rPIV3-2 and other chimeric PIV viruses of the invention *in vitro* is an important property for a live attenuated vaccine candidate that is
15 needed for large scale vaccine production. In contrast to rPIV3-2CT and rPIV3-2TM, rPIV3-1 was not attenuated *in vivo*. Thus, the chimerization of the HN and F proteins of PIV2 and PIV3 itself resulted in attenuation of replication *in vivo*, a novel finding for single-stranded, negative-sense RNA viruses. The mechanism for this host range restriction of replication *in vivo* is not known. Importantly, infection with these attenuated rPIV3-2CT
20 and rPIV3-2TM vaccine candidates induced a high level of resistance to challenge with PIV2 indicating that the antigenic structure of the chimeric glycoproteins was largely or completely intact. Thus rPIV3-2CT and rPIV3-2TM function as live attenuated PIV2 candidate vaccine viruses, exhibiting a desirable balance between attenuation and immunogenicity in both AGMs and hamsters.

The attenuating effects of the PIV3-PIV2 chimerization of the F and HN glycoprotein are additive with that specified by the *cp45* mutations. rPIV3-2 recombinants containing the *cp45* mutations were highly attenuated *in vivo* and provided incomplete protection in hamsters against challenge with PIV2 and little protection in AGMs. This is in
25 contrast to the finding with rPIV3-1*cp45* which was satisfactorily attenuated *in vivo* and protected animals against challenge with PIV1. The combination of the independent,
30 additive attenuating effects of the chimerization of PIV3-PIV2 glycoproteins and the 12 *cp45* mutations appeared too attenuating *in vivo*. Clearly, if the rPIV3-2CT and rPIV3-2TM vaccine candidates are found to be insufficiently attenuated in humans, the *cp45* attenuating

mutations should be added incrementally rather than as a set of 12 to achieve a desired balance between attenuation and immunogenicity needed for a live attenuated PIV2 vaccine for use in humans. The findings presented herein thus identify a novel means to attenuate a paramyxovirus and provide the basis for evaluation of these PIV3-PIV2 chimeric live
5 attenuated PIV2 vaccine candidates in humans. Importantly, the rPIV3-2CT or rPIV3-2TM viruses can also be used as vectors for other PIV antigens or for other viral protective antigens, e.g., the measles virus HA protein or immunogenic portions thereof.

Briefly summarizing the foregoing description and examples, recombinant chimeric PIVs constructed as vectors bearing heterologous viral genes or genome segments
10 have been made and characterized using a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and can be propagated in the same manner as if they were biologically-derived viruses. In preferred embodiments, recombinant chimeric human PIV (HPIV) vaccine candidates bear one or more major antigenic determinant(s) of a HPIV, preferably in a background that is attenuated by one or
15 more nucleotide modifications. Preferably, chimeric PIVs of the invention also express one or more protective antigens of another pathogen, for example a microbial pathogen. In these cases, the HPIV acts as an attenuated virus vector and is used with the dual purpose of inducing a protective immune response against one or more HPIVs as well as against the pathogen(s) from which the foreign protective antigen(s) was/were derived. As mentioned
20 above, the major protective antigens of PIVs are their HN and F glycoproteins. The major protective antigens of other enveloped viruses, for example viruses that infect the respiratory tract of humans, that can be expressed by the HPIV vector from one or more extra transcriptional units, also referred to as gene units, are their attachment proteins, e.g., the G protein of RSV, the HA protein of measles virus, the HN protein of mumps virus, or their
25 fusion (F) proteins, e.g., the F protein of RSV, measles virus or mumps virus. It is also possible to express the protective antigens of non-enveloped viruses such as the L1 protein of human papillomaviruses which could form virus-like particles in the infected hosts (Roden et al., *J. Virol.* 70:5875-83, 1996). In accordance with these teachings, a large array of protective antigens and their constituent antigenic determinants from diverse pathogens
30 can be integrated within chimeric PIV of the invention to generate novel, effective immune responses.

The present invention overcomes the difficulties inherent in prior approaches to vector based vaccine development and provides unique opportunities for immunization of

infants during the first year of life against a variety of human pathogens. Previous studies in developing live-attenuated PIV vaccines indicate that, unexpectedly, rPIVs and their attenuated and chimeric derivatives have properties which make them uniquely suited among the nonsegmented negative strand RNA viruses as vectors to express foreign proteins as vaccines against a variety of human pathogens. The skilled artisan would not have predicted that the human PIVs, which tend to grow substantially less well than the model nonsegmented negative strand viruses and which typically have been underrepresented with regard to molecular studies, would prove to have characteristics which are highly favorable as vectors. It is also surprising that the intranasal route of administration of these vaccines has proven a very efficient means to stimulate a robust local and systemic immune response against both the vector and the expressed heterologous antigen. Furthermore, this route provides additional advantages for immunization against heterologous pathogens which infect the respiratory tract or elsewhere.

These properties of PIV vectors are described herein above using examples of rPIV3 vectors which bear (i) a major neutralization antigen of measles virus expressed as a separate gene in wild type and attenuated backgrounds or (ii) major neutralization antigens of HPIV1 in place of the PIV3 neutralization antigens which express in addition a major neutralization antigen of HPIV2. These rPIV vectors were constructed using wild type and attenuated backgrounds. In addition, the description herein demonstrates the ability to readily modify the level of attenuation of the PIV vector backbone. According to one of these methods, varying the length of genome inserts in a chimeric PIV of the invention allows for adjustment of the attenuation phenotype, which is only apparent in wild type derivatives using very long inserts.

The present invention provides six major advantages over previous attempts to immunize the young infant against measles virus or other microbial pathogens. First, the PIV recombinant vector into which the protective antigen or antigens of measles virus or of other microbial pathogens is inserted is an attenuated rPIV bearing one or more attenuating genetic elements that are known to attenuate virus for the respiratory tract of the very young human infant (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., J. Infect. Dis. 171:1107-1114, 1995a; Karron et al., J. Infect. Dis. 172:1445-1450, 1995b). This extensive history of prior clinical evaluation and practice greatly facilitates evaluation of derivatives of these recombinants bearing foreign protective antigens in the very young human infant.

The second advantage is that the rPIV backbone carrying the measles HA or other protective antigen of another human pathogen will induce a dual protective immune response against (1) the PIV, for which there is a compelling independent need for a vaccine as indicated above, and (2) the heterologous virus or other microbial pathogen whose protective antigen is expressed by the vector. This contrasts with the VSV-measles virus HA recombinant described above which will induce immunity to only one human pathogen, i.e., the measles virus, and in which the immune response to the vector itself is at best irrelevant or is potentially disadvantageous. The coding sequences of the foreign genes inserted into various members of the *Mononegavirales* Order of viruses have remained intact in the genomes of the most of the recombinant viruses following multiple cycles of replication in tissue culture cells, indicating that members of this group of viruses are excellent candidates for use as vectors (Bukreyev et al., J. Virol. 70:6634-41, 1996; Schnell et al., Proc. Natl. Acad. Sci. U.S.A. 93:11359-65, 1996a; Singh et al., J. Gen. Virol. 80:101-6; Yu et al., Genes Cells 2:457-66, 1997).

Another advantage provided by the invention is that use of a human pathogen backbone, for which there is a need for a vaccine, will favor the introduction of such a live attenuated virus vector into an already crowded early childhood immunization schedule. In addition, immunization via the mucosal surface of the respiratory tract offers various advantages. A live attenuated PIV3 was shown to replicate in the respiratory tract of rhesus monkeys and to induce a protective immune response against itself in the presence of high quantities of maternally-acquired PIV3 antibodies. The ability of two candidate PIV3 vaccines to infect and to replicate efficiently in the upper respiratory tract of the very young human infant who possess maternally-acquired antibodies has also been demonstrated (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., J. Infect. Dis. 171:1107-1114, 1995a; Karron et al., J. Infect. Dis. 172:1445-1450, 1995b). This is in contrast to the currently licensed measles virus vaccine which is poorly infectious when administered to the upper respiratory tract of humans and which is highly sensitive to neutralization when administered parenterally to young children (Black et al., New Eng. J. Med. 263:165-169, 1960; Kok et al., Trans. R. Soc. Trop. Med. Hyg. 77:171-6, 1983; Simasathien et al., Vaccine 15:329-34, 1997). The replication of the HPIV vector in the respiratory tract will stimulate the production of both mucosal IgA and systemic immunity to the HPIV vector and to the expressed foreign antigen. Upon subsequent natural exposure to wild type virus, e.g., measles virus, the existence of vaccine-induced local and systemic

immunity should serve to restrict its replication at both its portal of entry, i.e., the respiratory tract, as well as at systemic sites of replication.

Yet another advantage of the invention is that chimeric HPIVs bearing heterologous sequences replicate efficiently *in vitro* demonstrating the feasibility for large scale production of vaccine. This is in contrast to the replication of some single-stranded, negative-sense RNA viruses which can be inhibited *in vitro* by the insertion of a foreign gene (Bukreyev et al., J. Virol. 70:6634-41, 1996). Also, the presence of three antigenic serotypes of HPIV, each of which causes significant disease in humans and hence can serve simultaneously as vector and vaccine, presents a unique opportunity to sequentially immunize the infant with antigenically distinct variants of HPIV each bearing the same foreign protein. In this manner the sequential immunization will permit the development of a primary immune response to the foreign protein which can be boosted during subsequent infections with the antigenically distinct HPIV also bearing the same or a different foreign protein or proteins, i.e., the protective antigen of measles virus or of another microbial pathogen. It is also likely that readministration of homologous HPIV vectors will also boost the response to both HPIV and the foreign antigen since the ability to cause multiple reinfections in humans is an unusual but characteristic attribute of the HPIVs (Collins et al., In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996).

Yet another advantage is that the introduction of a gene unit into a PIV vector has several unexpected, but highly desirable effects, for the production of attenuated viruses. First, the insertion of gene units expressing, for example, the HA of measles virus or the HN of PIV2 can specify a host range phenotype on the PIV vector that has not been previously recognized, i.e., the resulting PIV vector replicates efficiently *in vitro* but is restricted in replication *in vivo* in both the upper and lower respiratory tracts. These findings identify the insertion of a gene unit expressing a viral protective antigen as an attenuating factor for the PIV vector, a desirable property in live attenuated virus vaccines of the invention.

The PIV vector system has unique advantages over all other members of the single-stranded, negative-sense viruses of the Order Mononegavirales. First, most other mononegaviruses that have been used as vectors are not derived from human pathogens (e.g., murine HPIV1 (Sendai virus) (Sakai et al., FEBS Lett. 456:221-6, 1999), vesicular stomatitis

virus (VSV) which is a bovine pathogen (Roberts et al., J. Virol. 72:4704-11, 1998), and canine PIV2 (SV5) He et al., Virology 237:249-60, 1997)). For these nonhuman viruses, little or only weak immunity would be conferred against any human virus by antigens present in the vector backbone. Thus, a nonhuman virus vector expressing a supernumerary gene for a human pathogen would induce resistance only against that single human pathogen. In addition, use of viruses such as VSV, SV5, rabies, or Sendai virus as vector would expose vaccinees to viruses that they likely would not otherwise encounter during life. Infection with, and immune responses against, such nonhuman viruses would be of marginal benefit and would pose safety concerns, because there is little experience of infection with these viruses in humans.

An important and specific advantage of the PIV vector system is that its preferred, intranasal route of administration, mimicking natural infection, induces both mucosal and systemic immunity and reduces the neutralizing and immunosuppressive effects of maternally-derived serum IgG that is present in infants. While these same advantages theoretically are possible for using RSV as a vector, for example, we have found that RSV replication is strongly inhibited by inserts of greater than approximately 500 bp (Bukreyev et al., Proc. Natl. Acad. Sci. USA 96:2367-72, 1999). In contrast, as described herein, HPIV3 can readily accommodate several large gene inserts. The finding that recombinant RSV is unsuitable for bearing large inserts, whereas recombinant PIVs are highly suitable, represents unexpected results.

It might be proposed that some other viral vector could be given intranasally to obtain similar benefits as shown for PIV vectors, but this has not been successful to date. For example, the MVA strain of vaccinia virus expressing the protective antigens of HPIV3 was evaluated as a live attenuated intranasal vaccine against HPIV3. Although this vector appeared to be a very efficient expression system in cell culture, it was inexplicably inefficient in inducing resistance in the upper respiratory tract of primates (Durbin et al., Vaccine 16:1324-30, 1998) and was inexplicably inefficient in inducing a protective response in the presence of passive serum antibodies (Durbin et al., J. Infect. Dis. 179:1345-51, 1999). In contrast, PIV3 and RSV vaccine candidates have been found to be protective in the upper and lower respiratory tract of non-human primates, even in the presence of passive serum antibodies (Crowe et al., Vaccine 13:847-855, 1995; Durbin et al., J. Infect. Dis. 179:1345-51, 1999).

The use of PIV3 in particular as a vector offers yet additional advantages. For example, conditions have been established to obtain high titers of PIV3 in microcarrier culture that are 10 to 1000 times greater than can be achieved with viruses such as RSV and measles virus. Also, RSV infectivity is unstable, which complicates propagation, transport,
5 storage and handling. These problems will be obviated by development of a PIV-vectored RSV vaccine.

Importantly, two versions of PIV3 have undergone extensive clinical evaluation as candidate vaccines administered intranasally, namely BPIV3 and the attenuated HPIV3 cp45 strain. Each was found to be safe, immunogenic, and phenotypically
10 stable in children and infants. No other candidate engineered vector has been evaluated in children and infants, and in particular no other available vector has been evaluated for intranasal administration in this age group.

Another advantage of the PIV vector system is that, using HPIV3 as a model, a number attenuating mutations have been identified that can be introduced into the vector
15 backbone singly and in combination to obtain the desired degree of attenuation. For example, the specific mutations that confer the HPIV3 cp45 attenuation phenotype have been identified directly by sequence analysis and introduction into wild type recombinant virus. Additional attenuating mutations were developed by "importing" attenuating point mutations from Sendai virus and RSV. In some cases, it was possible to introduce certain
20 point mutations into recombinant virus using two nucleotide changes rather than one, which stabilizes the mutation against reversion to wild type. Ablation of expression of the C, D and V ORFs was shown to attenuate the virus. In addition, chimeric viruses of HPIV3 and bovine (B)PIV3 were developed to use the natural host range restriction of BPIV3 in primates as a means of attenuation. It also was found that certain sequence combinations
25 were attenuating, such as replacement of the HPIV3 HN and F ectodomains with their counterparts from HPIV2. Thus, a large menu of PIV attenuating mutations exists that can be used to attenuate the vector backbone as desired.

Thus, one aspect of the invention disclosed herein relates to a method of using selected recombinant PIVs as vectors to express one or multiple protective antigens of a
30 heterologous pathogen as supernumerary genes. The heterologous pathogens described herein include heterologous PIVs, measles virus, and RSV. In the examples above, rHPIV3 was engineered as a vector to express up to three separate supernumerary gene inserts each

expressing a different viral protective antigen. Furthermore, rHPIV3 readily accommodated a total aggregate insert length of at least 50% that of the wild type genome. Constructs were made with several different PIV vector backbones, namely: wild type HPIV3; an attenuated version of HPIV3 in which the N ORF was replaced by that of BPIV3; the HPIV3-1
5 chimeric virus, in which the HN and F ORFs of HPIV3 were replaced by their counterparts from HPIV1; a version of HPIV3-1 that was attenuated by the presence of three independent attenuating cp45 point mutations in the L gene; and a version of BPIV3 in which the HN and F genes were replaced by their counterparts from HPIV3. These vectors bearing one or more supernumerary genes replicated efficiently *in vitro*, demonstrating feasibility for their
10 commercial development, and they replicated and induced strong immune responses *in vivo* against both the vector and the inserts. In this way it is possible to construct a single recombinant PIV-based virus that is capable of inducing an immune response against at least four human pathogens, namely the PIV vector itself and the pathogens represented by the supernumerary genes.

15 A second aspect of the invention is to use the superior characteristics of PIV as a vaccine and as a vector to make a vaccine against RSV. RSV is a pathogen that grows less well than PIV, is unstable, and tends to induce immune responses that are poorly protective for reasons that are not completely understood. The development of a live-attenuated RSV vaccine has been underway for more than 35 years, indicating the difficulty
20 of achieving an appropriate balance between immunogenicity and attenuation for this human pathogen. Thus, there are compelling reasons for developing a live attenuated RSV vaccine that is not based on infectious RSV. The RSV major protective F and G antigens were expressed as supernumerary genes from a PIV vector, in this case BPIV3, obviating the need to produce a live-attenuated vaccine based on infectious RSV.

25 A third aspect of the invention described herein has been to develop PIV-based vectors bearing the antigenic determinants of different PIV serotypes. Since there is essentially no cross protection between serotypes, this makes it possible to develop a method for sequential immunizations with a common PIV vector in which the protective antigenic determinants are changed. Thus, a single attenuated PIV vector backbone such as derived
30 from rHPIV3, bearing supernumerary genes as desired, can be used for an initial immunization. A subsequent immunization, which preferably follows the first by 4-6 or more weeks, can be achieved using a version of the same PIV vector in which the vector glycoprotein genes have been replaced with those of a heterologous PIV serotype, such as in

